

# The Longevity of Residual Antibacterial Effect of Dentin Treated with Various Concentrations of Triple Antibiotic Paste

By:

Sarmad Mazin Alyas, DDS, MS

Submitted to the Graduate Faculty of the School of Dentistry in partial fulfillment of the requirements for the degree of Master of Science in Dentistry, Indiana University School of Dentistry, 2016

Thesis accepted by the faculty of the Department of Endodontics, Indiana University School of Dentistry, in partial fulfillment of the requirements for the degree of Master of Science in Dentistry.

---

Ghaeth Yassen  
Chair of the Research Committee

---

Ygal Ehrlich

---

Richard L. Gregory

---

Josef Bringas

---

Ned Warner

---

Kenneth J. Spolnik  
Endodontic Chair and Graduate  
Program Director

Date \_\_\_\_\_

## **ACKNOWLEDGMENT**

First and foremost, I would like to thank my parents, Nada and Mazin, for their continuous love, never ending support and for always believing in me. You have been my role models and motivation to always becoming a better person. Your daily encouragement, love, and words of wisdom are invaluable to me.

I would like to convey my deepest gratitude to Dr. Spolnik and the Indiana University Graduate Endodontic program for believing in me and giving me the opportunity to learn, train and grow professionally. My experience has been invaluable and I have enjoyed my residency and learned a great deal.

I would like to express the deepest appreciation to my supervisor, Dr. Yassen, for his contributions to this project. Thank you for your extensive knowledge and support throughout this research project. Your dedication and hard work laid the foundation for the present study. You were a pleasure to work with and I owe much of the credit for this project to your training and guidance. A special thank – you goes to Dr. Richard Gregory and Dr. Ygal Ehrlich. Without their guidance, this thesis would not have been possible. I am also grateful to rest of my committee members for all their help.

To my endodontic and dental school colleagues and faculty, you are an extension of a family to me. Dr. Spolnik, thank you for being the best program director and leading the department towards greatness. Dr. Bringas, thank you for teaching and guiding us in the clinic. It has been great to learn from you in Detroit and then again here in Indianapolis. Dr. Ehrlich, thank you for always keeping us on track and guiding our research projects. Dr. Warner, it has been great getting to know you this past year and learning from your experiences in private practice.

I would also like to thank all the part-time faculty members who take the time to come to



school to teach. Drs. Vail, Steffel, Newton, Hill, Deardorf, Hine, Berman, Prather, Adams, and Sahni, thank you all very much for making us stronger clinically and professionally. Thank you, Diane, for your administrative efforts, and for always keeping us organized and on track. Thank you to Linda, Danielle, Jenny, Steve, Karen and Elaine for assisting me every day in the clinic – you made my life so much easier.

Chris and Dan, I am so glad and lucky to have had you as my coresidents and couldn't have asked for better friends, colleagues and competitors. Being around you both, made me try harder and pushed me to be better with each case. Alex, Daryl, Jordon, Avni, Ben, and Scott, it was a pleasure being co-residents with all of you. I'm not sure residency would have been as much fun without all of you.

A special thanks to the Delta Dental Foundation and the AAE foundation for the grants awarded towards this project. Their financial contribution allowed us to complete this study.

## **TABLE OF CONTENTS**

Introduction.....	11
Review of Literature.....	17
Materials and Methods.....	47
Results.....	51
Figures .....	53
Discussion.....	73
Summary and Conclusions.....	79
References .....	81
Abstract.....	94
Curriculum Vitae.....	97

## **LIST OF ILLUSTRATIONS**

FIGURE 1.	Experimental design flowchart.	54
FIGURE 2.	Overview of specimen preparation: Each tooth was sectioned, cut to 4x4 mm, and the pulpal surface was flattened.	55
FIGURE 3.	Teeth were sectioned using a high-speed saw with water irrigation.	56
FIGURE 4.	The high-speed saw that was used with water irrigation (Lapcraft L'il Trimmer).	57
FIGURE 5.	The low-speed saw used with water irrigation (Isomet, Buhler).	58
FIGURE 6.	Each half-root was cut into a 4x4-mm square with a double-bladed low-speed saw with water.	59
FIGURE 7.	Dentin specimens (4x4mm) were mounted on Struer block for flattening and smoothing.	60
FIGURE 8.	The RotoPol 31 (bottom) / Rotoforce-4 (top) was used to flatten the bottom side and polish the pulpal side of the specimen.	61
FIGURE 9.	Dentin specimens after polishing and prior to sterilization.	62
FIGURE 10.	Dentin specimens were individually packaged and sterilized.	63
FIGURE 11.	Work station where dentin specimens were transferred into sterile 96 well plates prior to antibiotic treatment	64
FIGURE 12.	Dentin specimens placed in their perspective treatment groups. Starting from the top with group 1: 1000 mg/mL pure TAP; group 2: 100 mg/mL MC-TAP; group 3: 10 mg/mL MC-TAP; group 4: 1 mg/mL MC-TAP; group 5: MC only without TAP	65
FIGURE 13.	Dentin specimens after 3 weeks of treatment. Starting from the top with group 1: 1000 mg/mL pure TAP; group 2: 100 mg/mL MC-TAP; group 3: 10 mg/mL MC-TAP; group 4: 1 mg/mL MC-TAP; group 5: MC only without TAP; group 6: NaOCl	66
FIGURE 14.	Preparation of <i>E. faecalis</i> inoculum with BHI media	67
FIGURE 15.	Blood agar plates in incubator after spiral plating	68
Table 1	The mean and SE of log CFL/mL for bacterial biofilm grown for 3 weeks on dentin previously treated with different concentrations of TAP followed by immersion in PBS for 2 or 4 weeks.	69

FIGURE 16.	The mean $\pm$ SE of log CFL/mL for bacterial biofilm grown for 3 weeks on dentin previously treated with different concentrations of TAP followed by immersion in PBS for 2 weeks. Different upper case letters represent statistical significance between different treatments.	70
FIGURE 17.	The mean $\pm$ SE of log CFL/mL for bacterial biofilm grown for 3 weeks on dentin previously treated with different concentrations of TAP followed by immersion in PBS for 4 weeks. Different upper case letters represent statistical significance between different treatments.	71
FIGURE 18	The mean $\pm$ SE of log CFL/mL for bacterial biofilm grown for 3 weeks on dentin previously treated with different concentrations of TAP followed by immersion in PBS for 2 or 4 weeks. Different upper case letters represent statistical significance between different treatments within the same immersion time. Different lower case letters represent statistical significance between similar treatments within the two immersion times.	72

## **INTRODUCTION**

The roots of teeth typically take three years in order to fully develop.<sup>1</sup> Pulpal necrosis of immature teeth due to caries or trauma will stunt root development and can pose serious challenges in terms of tooth retention over time.<sup>2</sup> The compromised prognosis of these teeth is due to incomplete root formation, both in width and length, which increases the risk for cervical root fracture.<sup>3</sup> These immature teeth have thin walls with large canals, which makes cleaning and shaping of these systems technique sensitive. The blunderbuss shape and open apices of these teeth make conventional obturation more challenging as it is difficult to keep the obturation material from extruding beyond the apex.<sup>3, 4</sup>

Traditional apexification utilizing long-term calcium hydroxide has been used to address the open apices by creating a hard tissue apical barrier to adequately complete the root canal.<sup>5-7</sup> While apexification allowed for improved quality of obturation, the tooth remained fragile and root development remained arrested.<sup>8, 9</sup> Regenerative endodontic therapy has been utilized as an alternative treatment for necrotic immature teeth as it controls the infection in the tooth and allows for its continued development.<sup>10, 11</sup> The current recommended technique by the American Association of Endodontists consists of canal disinfection followed by induction of bleeding in that canal.<sup>12</sup> The blood from the apical area contains mesenchymal stem cells of the apical papilla, and the blood clot formed into the canal serves as a scaffold for these stem cells, while growth factors are obtained from the platelets or dentin.<sup>13-15</sup> This migratory process is believed to be responsible for tissue formation and the continuous development of the root. Numerous reports have shown evidence of success for regenerative treatment in immature necrotic



teeth in terms of increased root length and thickness, as well as resolution of infection.<sup>16,</sup>  
<sup>17</sup>

The most common medicaments and irrigation solutions used in canal disinfection during endodontic regeneration are calcium hydroxide ( $\text{Ca}(\text{OH})_2$ ), sodium hypochlorite ( $\text{NaOCl}$ ), and triple antibiotic paste (TAP).

$\text{NaOCl}$  is a powerful irrigant that is used commonly in endodontic therapy due to its germicidal and tissue dissolving abilities.<sup>18</sup> A low concentration of  $\text{NaOCl}$  (1.5%) is used in regenerative endodontic therapy due to its lack of cytotoxic effect on stem cells.<sup>19</sup>

TAP is the most commonly used medicament in endodontic regeneration, as more than 50% of the published cases have reported using TAP.<sup>20</sup> First reported by Hoshino et al, TAP is a mixture composed of metronidazole, ciprofloxacin, and minocycline.<sup>21</sup> Both metronidazole and ciprofloxacin prevent bacterial DNA synthesis; while minocycline binds to the 30S ribosomal subunit of bacteria to target protein synthesis.<sup>22, 23</sup> TAP is used due to its reported effectiveness against the pathogens found in the root canal system resulting in an increased ability to increase root canal wall thickness compared to  $\text{Ca}(\text{OH})_2$ .<sup>10</sup>

The inability to mechanically debride the canal system in order to effectively remove the biofilm in necrotic immature teeth resulted in the use of these antibiotic medicaments, which chemically interfere with bacterial growth. Therefore, the prevention of biofilm development, as well as the eradication or significant reduction of existing biofilm inside the canal, can be used as a measuring stick to determine the success of the antibiotic medicaments used.<sup>24</sup> The lack of filling in the canal as the regenerative tissue is developing may be conducive to bacterial proliferation. Thus, it is necessary to maintain

an aseptic environment in the pulp space following disinfection procedures for a longer period of time, to allow the new tissue sufficient time to establish itself in the root canal environment.<sup>25</sup>

While TAP has been proven as an effective antimicrobial medicament in the root canal system, TAP may negatively affect the chemical, physical and mechanical properties of radicular dentin.<sup>26</sup> It has been suggested that the use of TAP in the recommended clinical concentrations can be detrimental to the survival of human stem cells.<sup>27</sup> A recent study found that TAP had a greater cytotoxic effect than any of its three individual antibiotic components on both apical papilla (SCAPs) and human dental pulp cells (DPCs).<sup>28</sup> Furthermore, metronidazole was observed to be the only component of TAP that was not cytotoxic to either cell type. The use of diluted TAP has been suggested as a means to improve the survival rate of the stem cells.<sup>16</sup> However, excessive dilution of TAP might decrease the antibacterial effectiveness required for canal disinfection.

In order to obtain optimal results, it is important to achieve a balance between the antibacterial effect and the cytotoxic effects of TAP. Recent studies have suggested that TAP is directly cytotoxic to stem cells in levels above 0.1 mg/mL, and indirectly cytotoxic in levels above 1 mg/mL.<sup>29, 30</sup> As far as TAP's antibacterial property, one of the initial studies examined the effectiveness of TAP against bacteria taken from infected root dentin of canal walls. TAP in a concentration of 25 µg/mL was found to be effective in eliminating all the bacteria indicating that it can have bactericidal efficacy at this concentration.<sup>31</sup> Another report examined the effectiveness of TAP against newly formed *E. faecalis* and *Porphyromonas gingivalis* biofilm and compared it with calcium hydroxide. Furthermore, this report examined different concentrations of TAP and

calcium hydroxide against biofilm growth. The study demonstrated that TAP was more effective than calcium hydroxide against both strains of bacteria; moreover, TAP concentrations between 0.001-0.003 mg/mL were effective in reducing biofilm growth. This led to the concept that diluted TAP might have the required antibacterial efficacy during endodontic regeneration.<sup>21</sup> A recent study by the same group aimed to examine the antibacterial effect of different dilutions against 3-day old established *E. faecalis* biofilm. Solutions of 0.125, 0.25, 0.5, 1, and 10 mg/mL TAP dilutions were used.<sup>32</sup> This study indicated that while all the concentrations significantly reduced bacterial growth, only a concentration of 10 mg/mL of TAP was able to completely eradicate the biofilm.

The residual antibacterial activity (or substantivity), as defined in the literature, is the capability of the antibiotic to bind to the dentin structure and its subsequent release in an active form.<sup>33</sup> In a recent study, the residual antibacterial effect on *E. faecalis* inoculated dentin specimens treated with 1000, 1, and 0.5 mg/mL of TAP was explored.<sup>34</sup> The results indicated that dentin specimens treated with lower concentrations of TAP (1 and 0.5 mg/mL) had a significant residual antibacterial effect for up to 7 days while 1000 mg/mL of TAP had a significant residual antibacterial activity for up to 14 days. These results confirm previous studies regarding the presence of a residual antimicrobial activity in dentin treated with tetracycline and minocycline.<sup>35, 36</sup> TAP dilutions were used in liquid form in all previous studies, which is not applicable in a clinical situation where pastes are used. There has been no known study that has explored the residual antibacterial effects of low concentrations of TAP in a pasty consistency that can be applied clinically as an intracanal medicament.

Methylcellulose (MC) is the most commonly used vehicle to deliver commercial calcium hydroxide as an intracanal medicament.<sup>37</sup> A study has shown that MC-based calcium hydroxide was found to have higher retention capacity in radicular dentin when compared to the pure powder form of  $\text{Ca}(\text{OH})_2$ .<sup>38</sup> A similar concept can be proposed for TAP. Indeed, a recent study suggested that MC loaded with low concentrations of TAP and TAP caused 99% reduction in *E. faecalis* biofilm.<sup>34</sup> Therefore, the overall aim of this project has to determine the longevity of the residual antibacterial activity of dentin samples treated with various concentrations of MC-based TAP.

#### CLINICAL SIGNIFICANCE

Antibiotic pastes such as TAP are widely used in regenerative procedures. The ultimate goal of TAP is to adequately disinfect the canal. This study has two areas of clinical significance in Endodontics:

1. Develop a method of confidently and reliably applying TAP into the canal system by utilizing MC as a vehicle.
2. Provide baseline research for the residual antibacterial effect of the different concentrations of TAP necessary for the canal system, and the longevity of that effect.

#### SPECIFIC AIM:

To investigate the longevity of residual antibacterial activity of human radicular dentin treated with MC-based paste loaded with various concentrations of TAP on *E. faecalis* biofilm formation.

#### THE NULL HYPOTHESIS:

Radicular dentin treated with various dilutions of MC-based TAP will have no significant residual antibacterial effect on both tested time points.

THE ALTERNATIVE HYPOTHESIS:

Radicular dentin treated with at least one of the MC-based TAP concentrations will have a significant residual antibacterial effect after one or both tested time points.

## **REVIEW OF LITERATURE**

## HISTORY OF ENDODONTICS

The practice of dentistry has made some remarkable advancement and hasn't always been this way. In 5000 BC, ancient Sumerian texts attributed toothache to worms growing inside teeth and this was known as the "tooth worm" theory. This theory was not debunked until 1684 when Anton Von Leeuwenhoek microscopically observed microorganisms from tooth samples. In 1687, Charles Allen published the first book in the English language that was entirely devoted to dentistry. Although the book did not mention endodontics, it described different options for treating problematic teeth by method of transplantation, which involved removing the rotten teeth and stumps and replacing them with sound ones.<sup>39</sup>

In the 1700s, some methods were explored to find relief from pulpal and periapical disease while keeping one's tooth. These included incomplete treatment of the pulp with mechanical and chemical techniques and obturating the pulp chamber. Pierre Fauchard, who is regarded as the "founder of modern dentistry", was the first to present many of these ideas.<sup>39, 40</sup> In Fauchard's book, *The Surgeon Dentist*, he described many dental and endodontic concepts such as the creation of an access hole in an abscessed pulp chamber to allow it to drain. He also suggested obturating the chamber and canals using lead foil. This made Fauchard the first to discuss obturation in endodontics. Twenty-eight years later in Germany, Philip Pfaff discussed pulp capping using gold or lead in 1756.<sup>41</sup> Bourdet, in 1756, described endodontic therapy by method of intentional extraction followed by replantation in an attempt to save the nerve.<sup>39</sup>

In the middle of the 18<sup>th</sup> century, Robert Woofendale was the first to perform endodontic procedures in the United States. He cauterized the pulp with heated

instruments to alleviate pulpal pain. Woofendale also proposed the use of oil of cinnamon, cloves, turpentine, opium, and camphor to tackle pulpal pain.<sup>42, 43</sup> Later in the same 18<sup>th</sup> century, Frederick Hirsch described the use of percussion testing as a diagnostic method for periapical disease. He proposed a treatment protocol of inserting a heated probe into the pulp, in a similar fashion to Woofendal.<sup>40</sup>

The 19<sup>th</sup> century marked the emergence of the “vitalistic theory”. Between 1800-1850, the study of pulp and periradicular physiology became of interest to the dental community. They began to realize the importance and effects of pulp vitality and its associated treatment. During this time, pulpal anesthesia was introduced, as well as new instruments, which allowed for more efficient canal debridement.<sup>39</sup> J.B. Gariot introduced the concept of pulp vitality, and the ability to retain non-vital teeth, in 1805.<sup>44</sup> In 1809 Edward Hudson was the first to place gold foil into canal space.<sup>45</sup> Ten years following that, Charles Bew introduced the concept of pulpal circulation, with blood flow into the tooth pulp through the apical foramen and existing through the periodontal membrane.<sup>44</sup> In 1826 Leonard Koecker wrote *Principles of Dental Surgery*. In his book, he challenged the concept that a non-vital tooth could be maintained and claimed that removal of the pulp would cause the entire tooth to die, rendering it as a foreign object. Koecker, therefore, promoted prevention of necrosis with pulp capping procedures similar to that of Pfaff.<sup>43, 44, 46</sup> Only a few years later, SS Fitch described the “vitalistic theory” in his book *System of Dental Surgery* in 1829. He explained that just like hollow bone, the entire tooth is vital. The crown of the tooth was supplied by pulpal circulation, while the root was supplied by both pulpal circulation and periodontal ligament. Decoronation of crowns after pulpal extirpation was the result of this theory. The root was then left in the



socket and restored with a crown. The other school of thought advocated “nonvitalistic theory”. They believed that enamel and dentin are devoid of any circulation, sensibility and self-repair capabilities; hence pulp removal would not affect the tooth.<sup>44</sup> Once these concepts were defined, the decades to follow brought about the use of medicaments during endodontic treatment. In 1836 New York, Shearjashub Spooner introduced painless pulpal debridement to devitalize pulp before its removal by using arsenic trioxide. While arsenic trioxide caused severe damage to the periodontium, this technique became very popular due to its high success rate of reducing, and even eliminating, pain associated with endodontic procedures at the time.<sup>40, 47, 48</sup> In 1837 Jacob Linderer and his son, Joseph, explained the use of narcotic oil as a mean to anesthetize the pulp.<sup>41</sup> Edwin Maynard developed the first endodontic broach in 1938.<sup>49</sup> In 1839 Baker wrote the first complete account of root canal therapy, hence describing pulpal debridement, cleaning, and filling of the canals with gold foil.<sup>40</sup>

The second half of the 19<sup>th</sup> century unveiled new instruments, disinfectants, obturation materials, surgical endodontics, diagnostic tests, and prognostic factors. In 1850 creosote-soaked wood plugs were commonly used as a method to fill the canals.<sup>47</sup> In the same year, Codman described that the goal of pulp capping was to obtain a secondary dentin over the pulp.<sup>44</sup> A year later in 1851, Hullihen described the first endodontic surgical procedure. He defined methods of flap reflection, osteotomy, and trephination of the tooth to induce pulpal hemorrhage and reduce pain.<sup>43</sup> Thomas Rogers explained prognostic factors for pulp capping in 1857. A year later, Jonathan Taft stated that reparative dentin was more resistant to decay.<sup>41</sup> In 1864 Sanford Barnum introduced the rubber dam for the purpose of tooth isolation.<sup>47, 48</sup> In 1867, Dr. G. A. Bowman

introduced the modern obturation material of choice, gutta-percha.<sup>48</sup> At the same time, Clarke Dubuque introduced the use of hot gutta-percha as an obturation method.<sup>39</sup> Magitot introduced electric current for pulp testing in 1867.<sup>50</sup> G. V. Black recommended the use of zinc oxychloride as a capping material in 1870.<sup>40</sup> In 1878, Dr. G. O. Rodgers suggested that pathogenic microorganisms are the common cause of pulpal disease. In 1879, the septic theory began to replace the vitalism theory. It explained that infected teeth were the etiology of disease and the focus then shifted to disinfection as a treatment priority.<sup>44</sup> This led to the belief that an aseptic environment is a necessary aspect of endodontic treatment, in an effort to completely destroy and eliminate these pathogenic organisms.<sup>51</sup> In 1882, Arthur Underwood suggested the use of antiseptic agents in the pulp space to eliminate pathogens.<sup>39</sup> In the closing years of the 19<sup>th</sup> century, Dr. Bowman developed chloropercha, a combination of chloroform and gutta percha, which was used along with gutta-percha cones for obturation.<sup>47</sup>

In the early 1900s, groundbreaking advances in dentistry and Endodontics took place. These included the introduction of local anesthetics; the concept of focal infection theory was introduced and refuted; and the importance of canal length and apical size determination was realized. In 1905 Einhorn developed procaine (Novocaine) as an alternative to the previously used anesthetic agent, cocaine. The introduction of dental blocks as a method of anesthetizing was utilized at this time also.<sup>48, 49</sup> In 1913 the dental x-ray unit was introduced and became commercially available in 1919 after the advent of the Coolidge tube, which allowed for a more focused x-ray beam.<sup>52</sup> This expanded our understanding of the endodontic disease process as it allowed visualization of periapical radiolucencies associated with pulpal and periapical disease<sup>53</sup>. Radiographs also allowed

dentists such as G.V. Black to describe radiographic working length and apical gauging of a root canal system.<sup>51, 54</sup> This greatly enhanced our understanding of root canal anatomy and allowed for a more precise and accurate cleaning, shaping and sealing of the canal system.

During the early 20<sup>th</sup> century, the focal infection theory was introduced and placed the practice of endodontics under scrutiny. This theory claimed that various diseases could be caused or exacerbated throughout the body by the spread of microorganisms and their toxins that rise from a focus of infection (e.g. within the tooth).<sup>55, 56</sup> The rise of this theory was due to a lecture by William Hunter, a pathologist and physician at McGill University in Montreal, entitled “The Role of Sepsis and Antisepsis in Medicine” which was published in *Lancet* in 1911.<sup>52, 56</sup> Dr. Hunter stated that “gold fillings, gold caps, gold bridges, gold crowns, fixed dentures, built in, on, and around diseased teeth, form a veritable mausoleum of gold over a mass of sepsis to which there is no parallel in the whole realm of medicine or surgery.”<sup>55</sup> This propelled many physicians to recommend extraction of all endodontically treated or non-vital teeth, while some other physicians went as far as prescribing the extraction of all teeth for prevention of infection.<sup>56, 57</sup> Thankfully in the 1930s and 1940s, it was shown that there was not a clear cause-and-effect relation between dental and systemic diseases and infection. For instance, Logan distinguished between the presence of bacteria and infection in 1937.<sup>42</sup> During the same time, Tunick and Hammond identified microorganisms in pulps without disease.<sup>51, 54</sup> Burket reported in the same year that 200 unresolved arthritis cases, despite removal of infection foci, indicated that the relationship between foci and arthritis was not causative but rather associative.<sup>51</sup> The focal infection theory began to lose ground

in the medical and dental community as a result of these studies and this marked the beginning of the “scientific era.”<sup>52</sup>

During the middle of the 1900s, the use of antibiotics became prevalent. Specifically, Dr. Adams and Grossman introduced antibiotic use as an adjunct to root canal therapy in the 1940s.<sup>52</sup> Grossman recommended the use of non-aqueous Penicillin as an intracanal medicament. He later proposed the use of antibiotic impregnated paper points in the canal space to sterilize it.<sup>47</sup> This led to an interest in chemotherapeutic treatment of root canals; however, it was realized that antibiotics alone could not completely sterilize the canal space. This led to the concept of mechano-chemical preparation; the creation of a space mechanically to allow for the chemicals to disinfect the canal space.<sup>58</sup>

Organized endodontics was established in 1943 with the foundation of the American Association of Endodontists (AAE) in Chicago, Illinois. This led to the establishment of the specialty of endodontics in 1949, and as a result the American Board of Endodontics (ABE) was formed in 1956.<sup>59</sup> Through the hard work of its members and leaders, and as a result of the growing number of board certified specialists, the American Dental Association recognized endodontics as a specialty in 1963.<sup>52</sup>

The late 1900s and through the turn of the century introduced many exciting developments in the field of endodontics. Nickel titanium rotary files, microsurgical instruments, enhanced magnification and illumination, cone beam computed tomography, and more biocompatible materials such as mineral trioxide aggregate are all examples of this development that allowed for a more accurate diagnosis and a more predictable treatment.<sup>60-65</sup> During this era, the field of regenerative endodontics gained popularity.

2006 marked the first regenerative endodontics conference, which was held in Nova Southeastern University.<sup>66</sup> The AAE dedicated one-half million dollars to 29 regenerative projects at 13 different institutions between 2001 and 2010.<sup>67</sup> In 2012, the ADA Current Dental Terminology included a new code (D3354) for pulpal regeneration within the endodontic section.<sup>68</sup> This idea of regeneration is picking up so much attention and traction, that the Journal of Endodontics is dedicating an entire section in each volume towards new research and findings in regenerative dentistry.

## THEORY OF ENDODONTICS

The basis of all current endodontic theories and practices are formed on the Kakehashi, Stanley and Fitzgerald landmark study of 1965. The experiment demonstrated that open and exposed pulps of germ-free rats remained vital. Trauma from food impaction and exposure to oral cavity did not cause pulpal necrosis in these germ free rats; moreover, they were able to heal and maintain pulpal health. Conventional rats placed under identical conditions displayed inflammation, necrosis, and periapical pathosis.<sup>69</sup> In 1981, Moller et al. reported that periapical inflammation in monkeys resulted from infected pulp tissue, and not necrotic tissue alone.<sup>70</sup> Such studies underline the crucial role of microorganisms in the pathogenesis of pulpal and periapical pathology.

The objective for both primary and secondary endodontic infections is to achieve maximal reduction in the microbial load and their byproducts in order to prevent and to treat pulpal and periapical pathology, and to restore the function of the tooth.<sup>71-74</sup> Failure to achieve sufficient reduction in microorganisms and their byproducts could result in apical periodontitis, which is defined as destruction of the periodontium in the presence

or absence of symptoms.<sup>75</sup> Therefore, endodontic success is directly correlated with the reduction of pathogenic microorganisms.<sup>76</sup>

Classic authors such as Stewart, Grossman, and Schilder have described the important principles of successful endodontic therapy.<sup>71, 72, 77, 78</sup> In 1955, Stewart emphasized how endodontic therapy can be separated into three phases: chemomechanical preparation, microbial control, and obturation.<sup>77</sup> While microbial control must be considered throughout all treatment phases, it was the chemomechanical preparation that received the most attention. Grossman confirmed that chemomechanical preparation is needed to eliminate pathogenic bacteria and their toxins from the root canal system. He identified 13 principles of effective root canal therapy:

1. Aseptic technique.
2. Instruments should remain within the root canal.
3. Instruments should never be forced apically.
4. Canal space must be enlarged from its original size.
5. Root canal system should be continuously irrigated with an antiseptic.
6. Solutions should remain within the canal space.
7. Fistulas do not require special treatment.
8. A negative culture should be obtained before obturation of the root canal.
9. A hermetic seal of the root canal system should be obtained.
10. Obturation material should not be irritating to the periapical tissues.
11. If an acute alveolar abscess is present, proper drainage must be established.
12. Injections into infectious areas should be avoided.
13. Apical surgery may be required to promote healing of the pulpless tooth.

In 1967 Schilder discussed the ultimate object of root canal treatment is the elimination of diseased tissue and contents of the inflamed or infection pulp and periapical area. He highlighted the importance of chemomechanical preparation and introduced the three-dimensional obturation of the entire canal system.<sup>71</sup> These three authors among others were instrumental in identifying the three phases of root canal therapy, commonly known now as instrumentation, irrigation, and obturation.

## INSTRUMENTATION

It is regarded as the first phase of endodontic therapy. This process is defined as the enlargement of the canal-space all the way apically to permit access for the irrigating solutions.<sup>78-80</sup> The original shape of the canal must be maintained and instruments must not be forced apically beyond the canal space to prevent damage to the periodontium.<sup>78, 81</sup> While instrumentation reduced bacterial load drastically inside the canal, 35-53% of the canal walls remain untouched.<sup>82-84</sup> Moreover, bacteria typically penetrate into the dentinal tubules as well as lateral and accessory canals, which means only mechanical instrumentations won't be sufficient and irrigation is needed.<sup>85, 86</sup>

## IRRIGATION

During endodontic therapy, the canal should be continuously irrigated with antiseptic solutions, and those solutions must remain in the canal for a sufficient amount of time.<sup>87</sup> Due to its effectiveness as a broad spectrum antimicrobial agent as well as its ability to dissolve organic tissues, sodium hypochlorite (NaOCl) solution is recommended as the primary irrigation solution.<sup>88</sup> Due to its high pH of 11, NaOCl is

able to exert its antimicrobial activity through the activity of hypochlorous acid, which disrupts oxidative phosphorylation, membrane activities, and DNA synthesis.<sup>89-91</sup> Many factors influence the efficacy of NaOCl, such as temperature, concentration and exposure time, which happens to be all directly proportional to its degree of tissue dissolution and penetration into dentin tubules.<sup>87, 92, 93</sup> The inability to dissolve inorganic dentin particles in the smear layer is one drawback of NaOCl.<sup>94</sup> Furthermore, the smear layer may block the penetration of the NaOCl into the tubules.<sup>95</sup> Therefore, another solution was identified to address this; ethylenediamine tetra-acetic acid (EDTA).<sup>94</sup> One-minute of irrigation with EDTA is sufficient to remove the smear layer in the canal system.<sup>96</sup> Smear layer removal allows better penetration of the NaOCl and improves the fluid-tight seal after obturation of root canal systems.<sup>97, 98</sup> Another limitation of NaOCl is that it lacks substantivity and is ineffective against endotoxins.<sup>99-101</sup> Therefore, chlorhexidine gluconate (CHX) is used as a supplemental irrigation solution that has a broad-spectrum antimicrobial activity with sustained antimicrobial effects for up to 12 weeks.<sup>102, 103</sup> The cationic CHX's antimicrobial activity is exerted by electrostatically binding to bacteria and disrupting their cell wall.<sup>104, 105</sup> The formation of a harmful precipitate if mixed with NaOCl is one limitation of CHX. Initially, it was thought that this precipitate was Parachloroaniline (PCA) toxin; however, recent studies using NMR refuted this concept and instead suggested that it was parachlorophenylurea (PCU) and parachlorophenylguanidyl-1,6-diguanidyl-hexane (PCGH).<sup>106, 107</sup> Nevertheless, the formation of the precipitate should be prevented during endodontic therapy by flushing the canal with saline or alcohol between the two solutions.<sup>107</sup>



## OBTURATION

The final phase of endodontic therapy is obturation to achieve a hermetic seal of the canal. Obturation materials need to be biocompatible in order to not irritate the periapical tissues.<sup>78</sup> One systematic review concluded that a high success rate of obturation is achieved when the material is terminated 0 mm to 1 mm from the radiographic apex.<sup>108</sup> These results were confirmed with an outcome study that showed that obturation density and length are two significant prognostic factors for success of root canal therapy.<sup>109</sup> The use of endodontic sealer during obturation is extremely important to provide the best seal of the canal system.<sup>108</sup>

After successfully cleaning and obturation of the root canal system, a definitive restoration is needed to provide coronal seal and prevent bacterial contamination.<sup>110</sup> As seen, many steps are taken to reduce pathogenic microbial load in the tooth in order to have a successful outcome in root canal therapy.

## MICROORGANISMS

Endodontic microorganisms are typically classified into primary and secondary infectious bacteria. In endodontic infections, bacteria are present as a community known as biofilm. A biofilm is defined as a dynamic organization of complex biologic systems growing on a solid surface that provide bacteria with many advantages needed for their survival and virulence.<sup>110</sup>

In primary endodontic infections, the mixed flora of bacteria is predominately composed of gram-negative anaerobic rods.<sup>71, 111</sup> The microbial composition of infected immature teeth is similar to that of primary endodontic infections in permanent teeth with

an average number of species of 2.13 per root canal as shown by Nagata in 2014.<sup>112</sup>

*Actinomyces naeslundii*, a facultative, anaerobic, gram-positive rod is the most prevalent species found in the canals of immature teeth.<sup>112</sup> *A. naeslundii* is also found in the normal flora of caries, plaque and primary endodontic infections. It binds to salivary proteins, epithelial cells, and tooth surfaces.<sup>113, 114</sup> Its main virulence factor is correlated to activation of the innate immune system of the host cell, causing a release of cytokines and triggering inflammation.<sup>115-118</sup>

Another primary endodontic pathogen is *F. nucleatum*. It is a gram-negative, non-spore forming fusiform rod that is frequently found in patients with periodontal and periapical disease.<sup>118, 119</sup> Its essential role as a middle colonizer is crucial in oral biofilm development and formation. It allows several different gram positive and gram-negative bacteria to attach to its cell surface receptor. *F. nucleatum* is able to invade host tissue cells and modulate a host immune response.<sup>120</sup>

*P. gingivalis* is a gram negative black-pigmented bacterium that is an obligate anaerobe. It has been found in 50% of primary endodontic infections as well as the gingival sulcus, tongue and tonsils. The main virulence factors of *P. gingivalis* include lipopolysaccharide, fimbriae, capsule, and lipoproteins among many others.<sup>120-122</sup> Typically, a one-minute irrigation with 1% NaOCl is sufficient to remove *P. gingivalis* from the canal system.<sup>123</sup>

Some microbes not present during initial treatment can cause secondary endodontic infections by gaining access to the canal system during any point of root canal therapy.<sup>110, 121</sup> In secondary endodontic infections, the mixed flora of bacteria is

predominately composed of gram-positive facultative cocci with an average number of species of 1.3 per root canal.<sup>111, 124</sup>

One of the most common bacteria isolated from persistent and secondary endodontic infections is *E. faecalis*. It is a gram-positive, facultative anaerobe that is present in the oral cavity of individuals who have an endodontic history.<sup>125</sup> The prevalence of *E. faecalis* in persistent endodontic infections is due to its ability to invade dentinal tubules and resist the high pH of calcium hydroxide.<sup>110, 121, 124, 126</sup> Its main virulence factors include lytic enzymes, cytolysis, aggregation substance, pheromones, and lipoteichoic acid.<sup>127</sup> These virulence factors make *E. faecalis* a very stubborn bacterium in the canal system that is able to form a biofilm that makes it more resistant to phagocytosis, antibodies, and antimicrobials than non-biofilm organisms.<sup>128</sup> These microorganisms make endodontic therapy very challenging, especially in immature permanent teeth.

## IMMATURE NECROTIC TEETH

Despite a very high success rate that is associated with conventional endodontic therapy, immature permanent teeth with pulpal necrosis have not had similar success.<sup>129</sup> Due to the thin and short roots of immature necrotic teeth, they tend to have a compromised prognosis, which increases their risk for endodontic and restorative failure.<sup>3, 4</sup> Another challenge in immature necrotic teeth is obturation as it is associated with risks of overextension of filling material past the apex into the periradicular space.<sup>9</sup> Therefore, many treatment strategies have been developed and proposed to tackle immature permanent teeth.

## APEXIFICATION

In the 1960s, apexification was introduced as a procedure aimed to create a calcified barrier at the apex of a non-vital permanent tooth with an open apex by treating it with long-term calcium hydroxide.<sup>6</sup> This barrier will act as a matrix against which the operator can condense obturation material without extruding any of them past the apex. The apical bridge has been identified as osteoid or cementoid material, and has minute communications with the periapical tissues.<sup>6, 130</sup>

The procedure consists of access of the tooth to reach the canal space once rubber dam isolation has been established. Working length determination would precede minimal canal instrumentation. Disinfection in these cases was mostly achieved via irrigation. This is followed by placement of calcium hydroxide in the canal for several months. Typically, the treatment span could be 9 to 24 months but the patient is usually recalled at three-month intervals to determine apical barrier formation. Once adequate apical barrier has developed, the canal space is usually obturated with MTA or gutta percha and a definitive restoration is placed to seal the access.<sup>131</sup>

A limitation of apexification is the fact that it does not induce root wall length and thickness, even though closure of root apex is achieved. Another drawback of this protocol is the need for strict patient compliance over a long and very extended period of time. Due to the long-term use of calcium hydroxide and the thin and short nature of the root, these teeth are at a greater risk for cervical root fracture.<sup>8, 132-134</sup> The frequency of root fracture is mostly dependent on the stage of root development and can be as high as 77% as reported by Cvek.<sup>3</sup>

Recent endodontic advances have changed part of the apexification procedure. An artificial apical barrier technique has been adopted as an alternative to long-term calcium hydroxide use. The canal disinfection protocol is the same as described for the traditional apexification technique. Calcium hydroxide is then placed in the canal space for a short time of 1-2 weeks in an effort to raise the pH of the periapical tissues. The calcium hydroxide is rinsed from the canal once the tooth becomes symptom free. The canal is then dried, an artificial apical barrier made of a collagen membrane is placed at the apex and then an MTA apical plug of 4-mm thickness is condensed at the apex prior to coronal restoration.<sup>135</sup> The benefits of this technique include a significant reduction in treatment time, the ability to place a definitive restoration sooner, reduction of side effects associated with long-term treatment of calcium hydroxide, hence reducing the chances of coronal leakage or cervical root fracture. The success rate for this procedure is very high and ranges from 85-93.5%.<sup>136, 137</sup> However, just like traditional apexification, this technique does not allow for increased root length and thickness.

While modern apexification techniques have improved the prognosis for these teeth, the long-term prognosis remains compromised. Fortunately, the emergence of regenerative endodontic therapy has provided a better alternative with a more promising outcome for immature necrotic teeth.

## REGENERATIVE ENDODONTICS

Tissue engineering and regeneration has gained particular attention in many aspects of medicine and dentistry. It is defined as the development of biological substitutes that restore, maintain, replace or improve tissue function.<sup>138</sup> It is important to

distinguish healing by repair from healing by regeneration. Healing by repair occurs when the new tissue type is not identical or there is loss of structure or function. As opposed to healing by regeneration, which occurs when the new tissue type is identical to the tissue it is replacing, and when the structure and the function are completely restored.<sup>139</sup> Generally, three main factors are needed for tissue regeneration; those are stem cells, scaffolds, and growth factors.<sup>138</sup> Regenerative endodontics is an example of tissue regeneration. It is done by disinfecting the root canal system and inducing bleeding from the apical papilla, which usually contains stem cells.<sup>14, 140, 141</sup> This procedure aims to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex.<sup>142</sup>

## TERMINOLOGY

Regenerative procedures have been described by different terms.

“Revascularization” was one of the first terms used to describe this procedure. It is defined as restoration of the vascularity; it takes place as a natural physiologic process in all healing, whether by regeneration or repair.<sup>143</sup> Moreover, the newly generated tissue inside the canal is not always vascular; therefore, “revitalization” was proposed as an alternative term.<sup>16</sup> This was often misinterpreted to mean re-innervation. In an effort to describe the ideal goal of regeneration, the AAE has most recently adopted the term “regenerative endodontic procedures” (REPs).<sup>144</sup>

## History of Regenerative Procedures

Nygaard-Østby began to conceptualize regenerative endodontics in 1961. He tested whether healing can be promoted by the presence of a blood clot within a root canal system. Seventeen patients with necrotic or vital pulps received root canal therapy followed by enlargement of the apical foramen, placement of medicament for the necrotic teeth, and intracanal bleeding was evoked. Teeth were restored and followed for a period that ranged between 17 days to 3.5 years. The teeth were then extracted and histologically examined. All teeth showed resolution of symptoms of inflammation and pathosis. In some cases, radiographic evidence of apical closure was noted.<sup>145</sup> Moreover, an in-growth of connective tissue was seen histologically in some of the previously vital canals. This tissue was not identical to pulp tissue and lack desirable cells such as odontoblasts.<sup>16</sup> In 1966, a study was published that first reported the use of poly-antibiotic pastes to disinfect necrotic teeth and promote root development.<sup>16</sup> Myers treated infected mature and immature teeth in monkeys in 1974. Canals were disinfected with 5.25% NaOCl, apically enlarged the foramen and bleeding was evoked. While tissue growth was seen in many teeth after 24 weeks, it was usually accompanied by periapical inflammation and root resorption. One explanation could be improper disinfection of the canals or coronal leakage. This study also indicated that immature teeth responded better than the mature teeth as they demonstrated continued root growth and the largest amount of connective tissue growth in the canal.<sup>146</sup> Two years later, Nevins treated pulpless immature teeth in monkeys with biomechanical debridement followed by collagen-calcium phosphate gel for 12 weeks. Histologic examination demonstrated “revitalization” of the canal with various forms of connective tissue including “cementum, bone, and reparative dentin.”<sup>147</sup>

## RECENT DISCOVERIES

Recent research and discoveries have provided more insight into REPs. In 2001, Iwaya published the first contemporary regenerative endodontic case report, using a double antibiotic paste (DAP) composed of ciprofloxacin and metronidazole to treat an immature tooth with pulpal necrosis and a periapical lesion. Mechanical canal instrumentation was limited in an attempt to conserve potentially apical vital tissue that might aid in revascularization. Disinfection of the canal was mainly via irrigation with 5% NaOCl and 3% H<sub>2</sub>O<sub>2</sub> followed by DAP application. A layer of calcium hydroxide was then placed against the apical tissue and the access was restored with a definitive resin restoration. Continued root growth and apical closure was seen radiographically at 30-months.<sup>148</sup> A case report detailing successful treatment of a necrotic immature mandibular premolar using triple antibiotic paste (TAP), composed of ciprofloxacin, minocycline, and metronidazole, was published three years later by Banchs and Trope. They outlined a specific protocol for revascularization of immature necrotic teeth based on the healing observed in avulsed immature permanent teeth.<sup>149</sup> They hypothesized that if a similar environment could be created for necrotic immature teeth, revascularization may occur. This developed protocol was performed on a case that started with non-mechanical canal disinfection with 5.25-% NaOCl, Peridex, TAP for 4 weeks, and 5.25% NaOCl again. Following disinfection, apical bleeding was induced with an explorer into the canal to the level of the CEJ and left to clot for 15 minutes. MTA was then placed as a final seal. At the 2-year follow-up, complete resolution of symptoms was reported, root growth was observed, and the tooth responded positively to the cold test. Subsequently,



this protocol was repeated by many other practitioners and led to many successful case reports of regeneration.<sup>150-153</sup>

In 2005, Nakashima et al. defined the three requirements for REPs: stem cells, a scaffold, and growth factors.<sup>154</sup> Lovelace then quantified mesenchymal stem cells in the blood at the apical area in 2001. He concluded that the concentrations of the stem cells in the apical area were 600-fold greater than levels in the systemic blood.<sup>14</sup> Banchs and Trope explained that the blood clot serves as a scaffold for the growth of new tissue into the canal space.<sup>149</sup> Bose et al. quantified root development after REPs in 2009. He concluded that a 25.1-percent increase in width and 14.7-percent increase in length was noted with REPs.<sup>10</sup> A similar study was conducted in 2012 by Jeeruphan et al. and found a 28.2-percent increase in width and 14.9-percent increase in length.<sup>11</sup> Both of these studies conclude that tooth development associated with REPs is significantly more than observed for MTA or  $\text{Ca}(\text{OH})_2$  apexification in both of these studies. The ability of REPs to promote root growth as confirmed in 2014 when Kahler et al. reported a case series of 16 consecutive REPs and found resolution of the periapical pathosis in 90.3% and complete apical closure in 19.4% at 18 months.<sup>155</sup>

As far as the type of tissue found in REPs, many different findings were reported. In 2011 Yamauchi et al. found two types of new tissue associated with treated immature dog teeth: dentin-associated mineralized tissue [DAMT]) and bony islands (BI). The DAMT, devoid of any vasculature and less cellular in nature, was located near the dentinal wall. In contrast, the vascular and highly cellular bony islands were located in the canal lumen. In 2012, Wang et al. found three types of new tissue in treated immature dog teeth: intracanal cementum (IC), intracanal bone (IB), and other connective tissue. In

the same year, Shimizu et al. identified cells that resembled odontoblasts and loose pulp-like connective tissue. However, in 2013 Martin et al. performed a REP *in vivo* and noted mineralized tissue and fibrous connective tissue, but no pulp-like tissue or odontoblast-like cells.<sup>148, 149, 156-159</sup>

All of these studies had some key features in common that resulted in the early success reported for REPs. Most of the patients were young with large open apices. Minimal instrumentation was performed and disinfection relied heavily on sodium hypochlorite irrigation, calcium hydroxide or TAP interappointment medication, and the formation of a blood clot as a scaffold.<sup>160</sup> These case reports formed the foundation and lead to the development of the current protocols for REPs.

## INDICATIONS AND OUTCOMES FOR REPs

REPs have been mainly reserved for necrotic immature teeth of adolescents. This is due to the fact that a lot of the published trauma studies suggest apical diameters that are 1 mm or bigger have a higher likelihood of revascularization.<sup>161</sup> Laureys et al.; however, found that an apical foramen as small as 0.32 mm permitted revascularization in dog teeth. This suggests that size of the apical foramen may not be as important as previously thought.<sup>162</sup> Definition of success is what assesses the outcomes for REPs. The AAE has set three goals for measuring success: 1) periradicular healing and complete elimination of symptoms, 2) continued root growth, and 3) positive response to sensibility testing.<sup>12</sup> In order to achieve success, many studies have contributed to the development of the three pillars of REPs; disinfection, stem cells and growth factors, and presence of a scaffold.

## DISINFECTION & IRRIGATION

Kakehashi, Stanely and Fitzgerald established the foundation of any endodontic therapy through their experiment on germ-free rats.<sup>69</sup> REPs, like any other endodontic treatment, requires disinfection of the canal system. Thibodeau et al. proved this in 2007. They performed REPs on immature necrotic dog teeth and confirmed histologically that vital tissue were only present in teeth that were first disinfected.<sup>163</sup> The most common disinfection regimen starts by irrigation with NaOCl followed by an intracanal medicament with either calcium hydroxide or antibiotic pastes.<sup>151</sup>

### A) Sodium Hypochlorite (NaOCl):

Introduced by Coolidge in 1919, NaOCl remains the most commonly used irrigant in endodontics.<sup>164</sup> Its phenomenal antibacterial activity and capability to dissolve organic tissue makes it very desirable for treatment.<sup>114, 165, 166</sup> Concentrations of NaOCl have ranged from 0.5 – 8% in endodontic therapy. The lower concentrations, such as 1.5%, dissolve mainly necrotic tissue and minimize vital tissue destruction.<sup>167</sup> Although NaOCl solutions have powerful antimicrobial activity, they have several disadvantages in the context of REPs. NaOCl has a concentration-dependent cytotoxicity on stem cells. Moreover, this cytotoxicity negatively impacts stem cell attachment.<sup>144, 168, 169</sup> Also, NaOCl has been correlated with a reduction of the modulus of elasticity and flexural strength of dentine at 3% and 5% concentrations. For these reasons, a concentration of 1.5% NaOCl has been proposed and recommended for REPs during the disinfection phase and to avoid use during the induction of bleeding and stem cell phase.<sup>12</sup>

## B) Calcium Hydroxide ( $\text{Ca}(\text{OH})_2$ )

Introduced by Hermann in 1920, calcium hydroxide has been shown to propel its antimicrobial effect by inactivating and detoxifying lipopolysaccharide (LPS) endotoxin. LPS inactivation is crucial to limit the inflammatory response to the periapical tissue.<sup>110, 170, 171</sup> Calcium hydroxide is alkaline and works by direct contact with the bacteria. It releases hydroxyl ions, which create free radicals that inhibit DNA replication and bacterial cell activity. Moreover, the alkaline pH of 12.5 denatures some enzymes and structural proteins, which retards cellular metabolism of bacteria.<sup>172</sup> An in vitro study suggested that a 24-hour treatment with calcium hydroxide resulted in complete killing of enterococci.<sup>110, 173, 174</sup>

In regenerative endodontics calcium hydroxide has been approved, is widely available and has been supported in case series reports.<sup>11, 175</sup> Calcium hydroxide has also been shown to be conducive to stem cells of the apical papilla (SCAP) survival as it significantly increases the proliferation of SCAPs at a concentration of 1 mg/mL.<sup>29, 30</sup>

However, calcium hydroxide has some disadvantages. Pure calcium hydroxide can have some detrimental effects and should be avoided as it negatively affects the OPG/RANKL ratio and inhibits the formation of hard tissue.<sup>176</sup> Therefore, typically calcium hydroxide is mixed with sterile water or saline to form a slurry or paste that can be administered inside the canal system. In regards to REPs, Andreasen et al. reported that a four-week application decreased tooth fracture strength.<sup>8</sup> Similarly, Yassen et al. concluded that three-month application caused a significant reduction in root fracture resistance in extracted teeth and an increase in microhardness.<sup>177</sup> They also reported that

a one to four week application caused superficial collagen degradation.<sup>178</sup> Another limitation associated with calcium hydroxide is its antimicrobial effectiveness against certain endodontic pathogens; for instance it was found to be less effective than triple antibiotic paste against *E. faecalis* and *P. gingivalis* biofilm.<sup>21</sup>

### C) Triple Antibiotic Paste (TAP)

Endodontic infections are polymicrobial in nature. No single antibiotic agent can eliminate all the bacteria inside a canal, let alone, eradicate the biofilm layer.<sup>179</sup> First reported by Hoshino et al., TAP contains a combination of ciprofloxacin, metronidazole and minocycline.<sup>31, 180</sup> Both metronidazole and ciprofloxacin prevent bacterial DNA synthesis; while minocycline binds to the 30S ribosomal subunit of bacteria to target protein synthesis. TAP effectiveness could be due to the broad spectrum and bactericidal effect of metronidazole.<sup>22, 23</sup> *In vitro* studies have shown that 0.3 mg/mL of TAP is effective against the cultivatable bacteria of endodontic lesions.<sup>181</sup>

However, TAP comes with certain limitations in regards to REPs including discoloration, demineralization, and cytotoxicity. It is believed that the presence of minocycline causes discoloration of the dentin.<sup>156, 182</sup> Minocycline binds calcium ions via chelation, hence forming an insoluble complex that remains incorporated in the tooth matrix.<sup>183</sup> This chelating effect of minocycline, combined with the acidic pH (2.9) causes demineralization.<sup>178</sup> Lastly, stem cell cytotoxicity is a serious concern with TAP. The initial reported concentrations of TAP were as high as 1000 mg/mL. In high concentrations (1, 10, and 100 mg/mL), TAP has shown detrimental effects on SCAP.<sup>29</sup> Moreover, in 2012, Ruparel et al. found that direct exposure of 1 mg/mL TAP to SCAPs

caused the death of 50 percent of the cells ( $LC_{50}$ ). Therefore, reduced concentrations of TAP have been recommended.<sup>12</sup> On a side note, disinfection with 2% chlorhexidine has been shown to cause severe cytotoxicity to SCAPs and is therefore also contraindicated in REPs.<sup>109</sup>

Another disadvantage of TAP is its application and lack of availability. If a paste is formulated, it is important to let the parents know that it is not FDA approved and must be made by a compounding pharmacy.

#### D) Ethylenediaminetetraacetic acid (EDTA)

Ethylenediaminetetraacetic acid (EDTA) is a chelating agent used to remove the inorganic portion of the smear layer and was first described by Ferdinand Munz in 1935.<sup>95</sup> This smear layer consists of debris generated by mechanical instrumentation combined with bacterial remnants and byproducts. The smear layer typically clogs dentinal tubules inside the canal system. EDTA is able to sequester di- and tricationic metal ions, such as  $Ca^{2+}$  and  $Fe^{3+}$ . Moreover, EDTA can actually cause bacterial death when there is direct exposure of the EDTA to surface proteins over an extended period of time.<sup>110</sup> In REPs context; EDTA is thought to improve the environment for regeneration by several mechanisms.

A study has shown that irrigation with 17% EDTA leads to the removal of the smear layer thereby exposing dentin tubules. These exposed tubules have been shown to facilitate the release of growth factors from dentin.<sup>184-186</sup> It has also been shown that EDTA is able to increase dentin surface roughness, which might increase adherence of stem cells to dentin.<sup>187</sup> In addition, dental pulp stem cells have demonstrated an intimate

association with dentin that has been pre-treated with EDTA.<sup>15</sup> Lastly, EDTA has been shown to reverse the cytotoxic effects of NaOCl partially, thus increasing the survival of SCAPs.<sup>169</sup> Although EDTA provides several benefits for REPs, its main disadvantage is erosion of peritubular and intertubular dentin when applied for 10 minutes as observed with SEM.<sup>96</sup>

## STEM CELLS

Stem cells are a very integral aspect of tissue engineering and REPs. Adult stem cells can either be multipotent or pluripotent; meaning that they can either divide into another cell like itself or the stem cells can develop into any human cell. Lovelace found that a major reservoir of stem cells is present in the periapical area of necrotic immature teeth. When bleeding is evoked, mesenchymal stem cells are delivered to the canal space.<sup>14</sup> These cells will then differentiate into various pulpal cells such as stem cells from apical papilla (SCAPs), dental pulp stem cells (DPSC), dental follicle progenitor stem cells (DFPCs), periodontal ligament stem cells (PDLSCs), and stem cells from human exfoliated deciduous teeth (SHEDs).<sup>141, 188-192</sup> Endodontic regeneration stem cells are postnatal and have shown a great deal of promise.<sup>193</sup> Stem cells are concentrated in the cell-rich zone of the pulp, near the odontoblastic layer.<sup>131</sup> The five types of cells described have shown promise for use in regenerative endodontic procedures and are essential for pulp fibroblasts, extracellular matrix, and collagen regeneration.<sup>131, 190, 193</sup> Also, it has been shown that the dental pulp has regenerative properties to assist in regeneration.<sup>154, 194</sup> While it has been shown that DPSCs can differentiate into odontoblasts after carious pulp exposure,<sup>178</sup> it is believed that SCAP are the main source

of undifferentiated cells in the process of root development.<sup>188</sup> This is mainly because of their superior proliferation rates to DPSCs and have previously differentiated to odontoblastic-like cells resulting in new production of dentin *in vivo*.<sup>141, 188, 195</sup>

## SCAFFOLD

Scaffolds are the third aspect of the regenerative trifecta. The function of a scaffold is to serve as an extracellular matrix that allows for transport of nutrients, oxygen, and metabolic waste to the site.<sup>154</sup> In 1976, Nevins was the first to introduce the use of a collagen gel scaffold in regenerative endodontic procedures.<sup>147</sup> A study by Thibodeau et al. suggested that the blood clot plays an important role in REPs. It showed that groups with blood clots as a scaffold, as opposed to collagen, had a better success rate.<sup>163</sup> A study by Hutmacher identified six properties of an ideal scaffold for REPs:<sup>147</sup>

1. Porous structure for tissue and vascular integration.
2. Biodegradable at a rate of tissue formation.
3. Allow cellular attachment for differentiation and proliferation.
4. The mechanical properties of the site being implanted must be adequate.
5. Does not elicit any adverse reactions.
6. Easily formed into different sizes and shapes.

While the blood clot from lacerated periapical tissue has traditionally served as the scaffold in REPs, other scaffolds are under investigation.<sup>142</sup> Numerous case reports have indicated success using platelet rich plasma (PRP) or platelet rich fibrin (PRF) as a scaffold.<sup>150, 196-198</sup> Several authors have also identified growth factors that are released from these scaffolds.<sup>15, 184, 185</sup>



## GROWTH FACTORS

In addition to the growth factors that are already present in the dentin, others have shown promise in REPs. For example; it was shown that long-term corticosteroid use resulted in reduction of pulp chamber.<sup>173</sup> A study showed that dexamethasone increased the differentiation of human dental pulp cells into odontoblast-like cells, and this effect was exacerbated when it was combined with 1,25-dihydroxyvitamin D3.<sup>171, 174</sup> Growth factors are known to positively affect regeneration and there is much to learn about their interactions and exact implications in REPs.

## RECOMMENDED GUIDELINES FOR REPs

As a result of continued research and development in the field of REPs, the AAE has published certain recommendations and guidelines for REPs. The following is a copy of those considerations as they were revised and updated by the AAE on 4/12/2015:<sup>12</sup>

### Case Selection:

- Tooth with necrotic pulp and an immature apex.
- Pulp space not needed for post/core, final restoration.
- Compliant patient/parent.
- Patients not allergic to medicaments and antibiotics necessary to complete procedure (ASA 1 or 2).

### Informed Consent

- Two (or more) appointments.

- Use of antimicrobial(s).
- Possible adverse effects: staining of crown/root, lack of response to treatment, pain/infection.
- Alternatives: MTA apexification, no treatment, extraction (when deemed nonsalvageable).
- Permission to enter information into AAE database (optional).

#### First Appointment

- Local anesthesia, dental dam isolation and access.
- Copious, gentle irrigation with 20 ml NaOCl using an irrigation system that minimizes the possibility of extrusion of irrigants into the periapical space (e.g., needle with closed end and side-vents, or EndoVac™). Lower concentrations of NaOCl are advised [1.5% NaOCl (20 mL/canal, 5 min) and then irrigated with saline or EDTA (20 mL/canal, 5 min), with irrigating needle positioned about 1 mm from root end, to minimize cytotoxicity to stem cells in the apical tissues.
- Dry canals with paper points.
- Place calcium hydroxide or low concentration of TAP. If TAP is used: 1) consider sealing pulp chamber with a dentin bonding agent [to minimize risk of staining] and 2) mix 1:1:1 ciprofloxacin: metronidazole: minocycline to a final concentration of 0.1 mg/ml.
- Deliver into canal system via syringe
- If triple antibiotic is used, ensure that it remains below CEJ (minimize crown staining).

- Seal with 3-4 mm of a temporary restorative material such as Cavit™, IRM™, glass ionomer or another temporary material. Dismiss patient for 1-4 weeks.

#### Second Appointment (1-4 weeks after 1st visit)

- Assess response to initial treatment. If there are signs/symptoms of persistent infection, consider additional treatment time with antimicrobial, or alternative antimicrobial.
- Anesthesia with 3% mepivacaine without vasoconstrictor, dental dam isolation.
- Copious, gentle irrigation with 20 ml of 17% EDTA.
- Dry with paper points.
- Create bleeding into canal system by over-instrumenting (endo file, endo explorer) (induce by rotating a pre-curved K-file at 2 mm past the apical foramen with the goal of having the entire canal filled with blood to the level of the cemento–enamel junction). An alternative to creating a blood clot is the use of platelet-rich plasma (PRP), platelet rich fibrin (PRF) or autologous fibrin matrix (AFM).
- Stop bleeding at a level that allows for 3-4 mm of restorative material.
- Place a resorbable matrix such as CollaPlug™, CollaCote™, CollaTape™ or other material over the blood clot if necessary and white MTA as capping material.
- A 3–4 mm layer of glass ionomer (e.g., Fuji IILC™, GC America, Alsip, IL) is flowed gently over the capping material and light-cured for 40 s. MTA has been associated with discoloration. Alternatives to MTA should be considered in teeth where there is an esthetic concern.

- Anterior and Premolar teeth - Consider use of CollaTape/CollaPlug and restoring with 3 mm of RMGI followed by bonding a filled composite to the beveled enamel margin.
- Molar teeth or teeth with PFM crown - Consider use of CollaTape/CollaPlug and restoring with 3 mm of MTA, followed by RMGI or alloy.

#### Follow-up

- Clinical and Radiographic exam
  - No pain, soft tissue swelling or sinus tract (often observed between first and second appointments).
  - Resolution of apical radiolucency (often observed 6-12 months after treatment)
  - Increased width of root walls (this is generally observed before apparent increase in root length and often occurs 12-24 months after treatment).
  - Increased root length.
  - Positive pulp vitality test response
- The degree of success of regenerative endodontic procedures is largely measured by the extent to which it is possible to attain primary, secondary, and tertiary goals:
  - Primary goal: The elimination of symptoms and the evidence of bony healing.
  - Secondary goal: Increased root wall thickness and/or increased root length (desirable, but perhaps not essential)

- Tertiary goal: Positive response to vitality testing (which if achieved, could indicate a more organized vital pulp tissue)

## **MATERIALS AND METHODS**

### ***Human teeth selection:***

Extracted human teeth were collected with IRB approval (Study #: 31325524) and stored in 0.1% thymol at 4°C. Inclusion criteria were as follows: caries-free, complete root formation, and at least 4 mm midroot diameter in either buccolingual or mesiodistal direction. Exclusion criteria were as follows: caries or restorations, hypocalcification, hypoplasia, cracks, incompletely formed roots.

### ***Specimen preparation:***

Human teeth that met the initial inclusion/exclusion criteria were prepared into 120 dentin specimens. Teeth were removed from 0.1% thymol and rinsed in deionized (DI) water for 10 seconds. Teeth were decoronated and the roots were sectioned buccolingually using a high-speed saw with water irrigation (Fig. 1-4). The inside of each half-root was flattened manually using a coarse vertical polishing wheel without water until the root canal concavity was no longer visible. Each half-root was secured to a thick acrylic plate with sticky wax and cut to a 4x4mm dimension with a double-bladed low speed saw with water (Fig. 5, 6). Specimens were secured to a 38 mm cylindrical mounting block with sticky wax (Fig. 7, 8). The pulpal side of the dentin specimen were sequentially polished with 500, 1200, and 2400 grit SiC abrasive papers using a Struers Rotapol 31/Rotoforce 4 polishing unit. Then, specimens were polished for 3 minutes using a polishing pad at 150 rpm with 1 µm diamond polishing suspension (Struers Inc). After preparation, specimens were re-examined and excluded if they contain any surface defects (Fig 9, 10). Specimens were sonicated with 5.25% NaOCl and 17% EDTA for 4 minutes to remove the smear layer.<sup>199, 200</sup>

### ***Medicament preparations***

Pastes loaded with various concentrations of TAP were prepared as described by Prather et al.<sup>37</sup> and Yassen et al.<sup>201</sup> In summary, 2500, 250 and 25 mg of antibiotic powders comprising equal portions of minocycline, metronidazole and ciprofloxacin (Champs Pharmacy, San Antonio, TX, USA) were dissolved in 25 mL of sterile water, respectively. Then, 2 g of methylcellulose powder (Methocel 60 HG; Sigma-Aldrich, St Louis, MO, USA) was added to each TAP solution under magnetic stirring to obtain a

homogenous paste with TAP concentrations of 100, 10 and 1 mg/mL. A placebo paste with no antibiotics was prepared utilizing the same method. Furthermore, an additional control group was treated with 1.5% NaOCl without any medicament.

***Treatment of the dentin samples:***

Dentin specimens were sterilized in ethylene oxide and specimens were placed independently inside wells of a sterile 96 well plate with the pulp surface facing upward to receive the treatment. Dentin samples were randomized into six treatment groups (n=20) (Figure 2). Group one was treated with pure 1000 mg/mL of TAP as a positive control. Each sample in the next three groups was treated with a 0.2 mL of diluted MC-based TAP (100, 10 mg/mL, and 1 mg/mL). Group five was treated with the same volume of MC placebo paste, and group six was treated with 1.5% NaOCl (Fig. 11, 12). The paste-treated dentin samples (groups 1-5) were incubated for 3 weeks at 37°C with approximately 100% humidity. To maintain the humid environment inside the wells and prevent the treatment pastes from drying out during incubation, all the empty wells of the 96 well plates were filled with sterile DI water. The samples in the sixth group received no paste treatment and was irrigated with 10 mL of 1.5% NaOCl for five minutes. Then, all specimens were removed, rinsed with DI water for 3 minutes, and irrigated for five minutes with 17% EDTA. The 20 dentin samples from each group were then sub-divided into two subgroups (n=10) and tested for residual antibacterial effect either 14 or 30 days after antibiotic removal by incubating each dentin sample in 0.2 mL of PBS at 37°C until the allocated time of antibacterial testing (Fig. 13).

***Bacterial strain and media***

*E. faecalis* (ATCC 29212) was grown initially on anaerobic blood agar plates (CDC, BioMerieux, Durham, NC). Additionally, brain heart infusion broth with 5 g/l yeast extract (BHI-YE) was utilized to grow the bacterium in 5% CO<sub>2</sub> environment at 37°C (Fig. 14).

***Inoculation with bacteria:***



Fresh BHI-YE growth media (190 µl) inoculated with 10 µl of an overnight *E. faecalis* culture ( $10^6$  colony forming units (CFU/mL))<sup>21</sup> was added to each dentin specimen and incubated aerobically for 3 weeks at 37°C in 5% CO<sub>2</sub>. The BHI-YE was replaced every three days during this period.

### ***Measuring growth:***

After the inoculation period of 3 weeks, the dentin specimens from all treatment groups were placed individually in plastic test tubes containing 200 µl of sterile saline, sonicated for 30 seconds, and vortexed for 30 seconds to detach biofilm cells. The detached biofilm cells were diluted 1:100 and 1:10,000 in sterile saline, spirally plated on blood agar plates (CDC, BioMerieux) and incubated for 48 h in 5% CO<sub>2</sub> at 37°C (Fig. 15). The number of CFUs/mL was evaluated using an automated colony counter (Synbiosis, Inc., Frederick, MD).

### ***Statistical methods:***

Summary statistics (mean, standard deviation, standard error, range) were calculated for each study outcome by group. Two-way ANOVA was used to test the effects of concentration and duration of residual antibacterial on dentin treated with TAP. Additionally, pair-wise comparisons between groups was made using Fisher's Protected Least Significant Differences to control the overall significance level at 5%.

### ***Sample size:***

Based on the pilot study, the within-group standard deviation is expected to be 1.7. With a sample size of 10 per group for each time point, the study has 80% power to detect a difference of 2.3 between any two groups for log CFU/volume, assuming two-sided tests conducted at a 5% significance level for each test.

## RESULTS

The first time point examined dentin samples that were incubated in PBS for 2 weeks after treatment with TAP or controls for three weeks. Statistical comparison of the different tested dilutions demonstrated a significant reduction in *E. faecalis* growth on dentin samples treated with TAP concentrations of 10, 100 and 1000 mg/mL compared to 1 mg/mL of TAP, MC and NaOCl ( $P = 0.0001$ ). There were no significant differences in bacterial growth among dentin treated with MC, NaOCl and 1 mg/mL TAP. The mean log bacterial growth for 1 mg/mL was 6.59 CFU/mL. The mean log bacterial growth for MC and NaOCl were 6.82 and 6.52 CFU/mL, respectively. There was no bacterial growth on dentin samples treated with TAP dilutions of 10, 100 or 1000 mg/mL (Table 1, Fig. 16).

The second time point examined dentin samples that were incubated in PBS for 4 weeks following the 3 weeks of treatment with TAP or controls. The four dentin groups treated with various concentrations of TAP demonstrated significant reduction in bacterial growth in comparison to dentin treated with MC and NaOCl ( $p < 0.5$ ). Dentin treated with 100 mg/mL of TAP demonstrated significant reduction in biofilm formation in comparison to all other groups ( $p < 0.01$ ). Dentin treated with 1000 mg/mL demonstrated significant reduction in biofilm formation in comparison to that treated with 10 mg/mL of TAP, 1 mg/mL of TAP, MC and NaOCl ( $p < 0.05$ ). Dentin treated with 10 mg/mL of TAP induced significant reduction in biofilm formation in comparison to that treated with 1 mg/mL of TAP, MC and NaOCl ( $p < 0.05$ ). Dentin treated with 1 mg/mL of TAP induced significant reduction in biofilm formation in comparison to that treated with MC and NaOCl ( $p < 0.05$ ). Dentin samples treated with 100 mg/mL of TAP had no bacterial growth. On the other hand, all remaining treatment groups had bacterial growth. Dentin samples treated with 1000 mg/mL TAP had a mean log value of 1.04 CFU/mL. Samples treated with 10 mg/mL of TAP had a mean log value of 3.15 CFU/mL. Dentin samples treated with 1 mg/mL TAP had a mean log value of 6.54 CFU/mL. The MC and NaOCl control groups had mean log values of 7.24 and 7.25 CFU/mL, respectively (Fig. 17).

When comparing both time points, bacterial growth was significantly lower on dentin samples that were incubated for 2 weeks than those at 4 weeks for 10 mg/mL ( $p = 0.0001$ ), 1000 mg/mL ( $p = 0.0335$ ), MC ( $p = 0.0273$ ), and NaOCl ( $p = 0.0027$ ) treatments

(Table 1). There was no significant difference between the two time points for samples treated with 1 mg/mL ( $p=1.00$ ) or 100 mg/mL ( $p=1.00$ ) (Fig. 18).

**FIGURES AND TABLES**

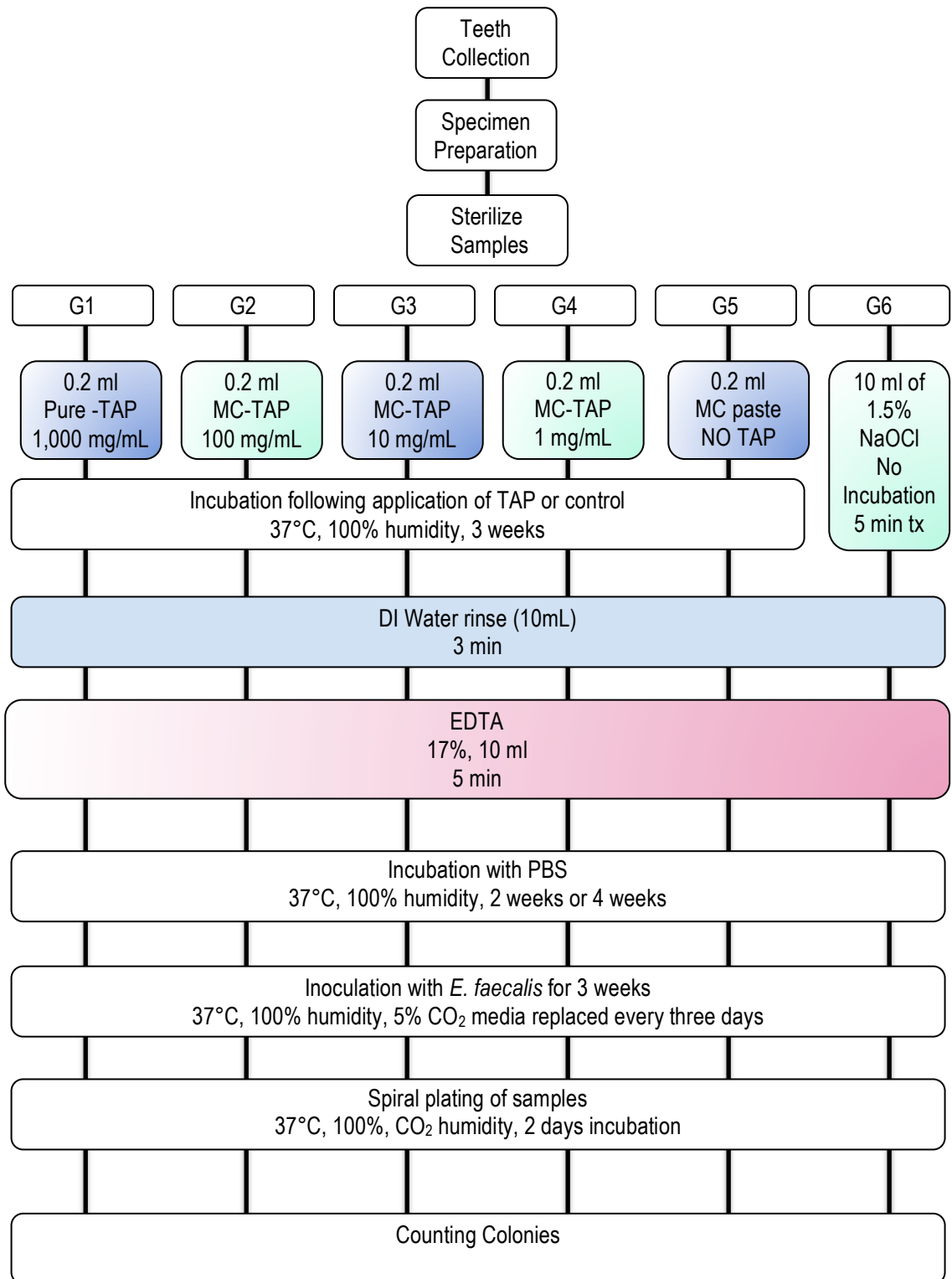


FIGURE 1.

Experimental design flowchart depicting the steps and sequence and design of the study.

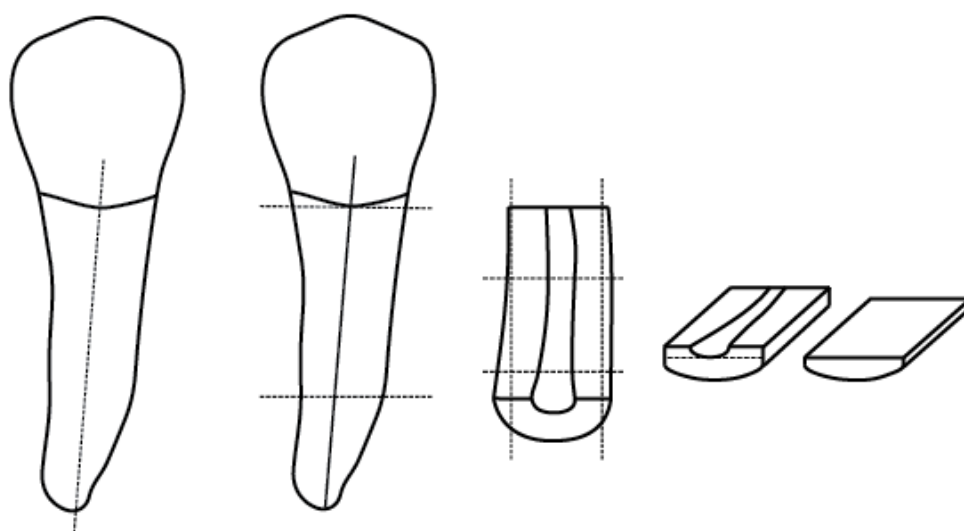


FIGURE 2.	Overview of specimen preparation: Each tooth was sectioned, cut to 4x4 mm, and the inner surface was flattened.
-----------	---

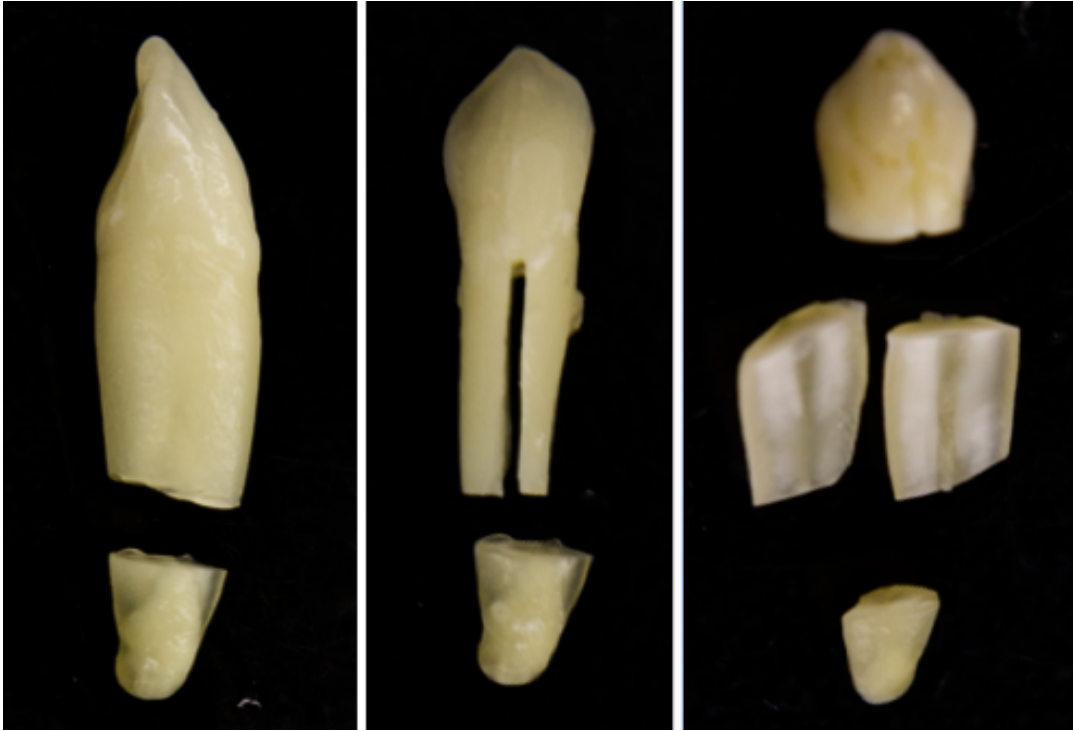


FIGURE 3.	Teeth were sectioned using a high-speed saw with water irrigation.
-----------	--





FIGURE 4.	The high-speed saw that was used with water irrigation (Lapcraft L'il Trimmer).
-----------	---

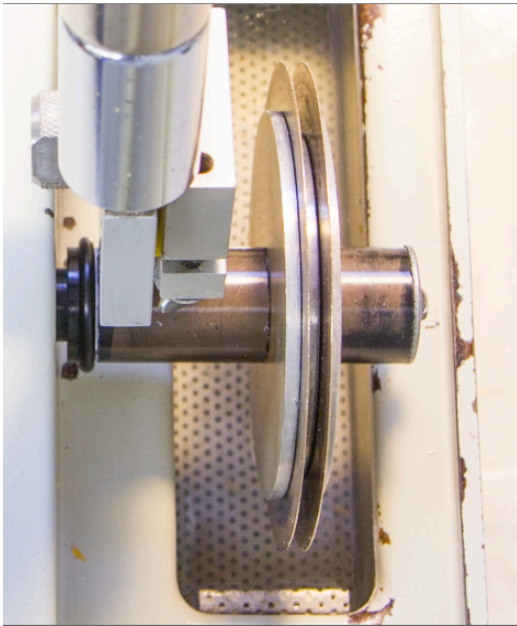


FIGURE 5	The low-speed saw used with water irrigation (Isomet, Buhler).
----------	--

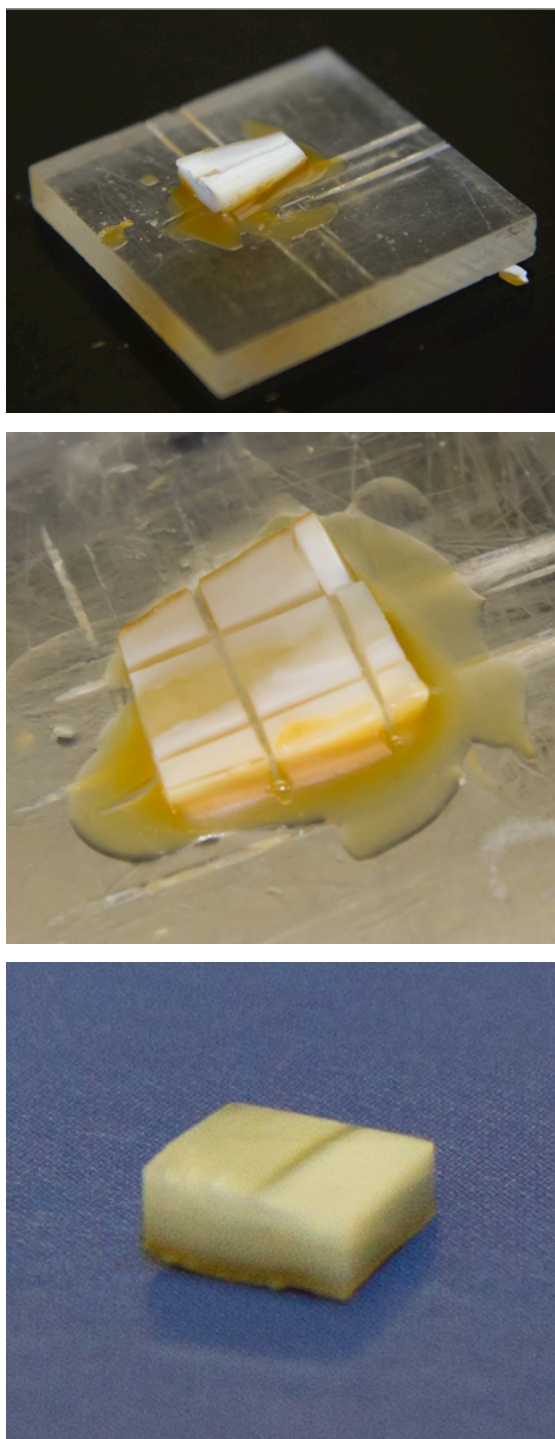


FIGURE 6.	Each half-root was cut into a 4x4-mm square with a double-bladed low-speed saw with water.
-----------	--



FIGURE 7.	Dentin specimens (4x4mm) were mounted on Struer block for flattening and smoothing.
-----------	---



FIGURE 8. The RotoPol 31 (bottom) / Rotoforce-4 (top) was used to flatten the bottom side and polish the pulpal side of the specimen.





FIGURE 9

Dentin specimens after polishing, prior to steralization.

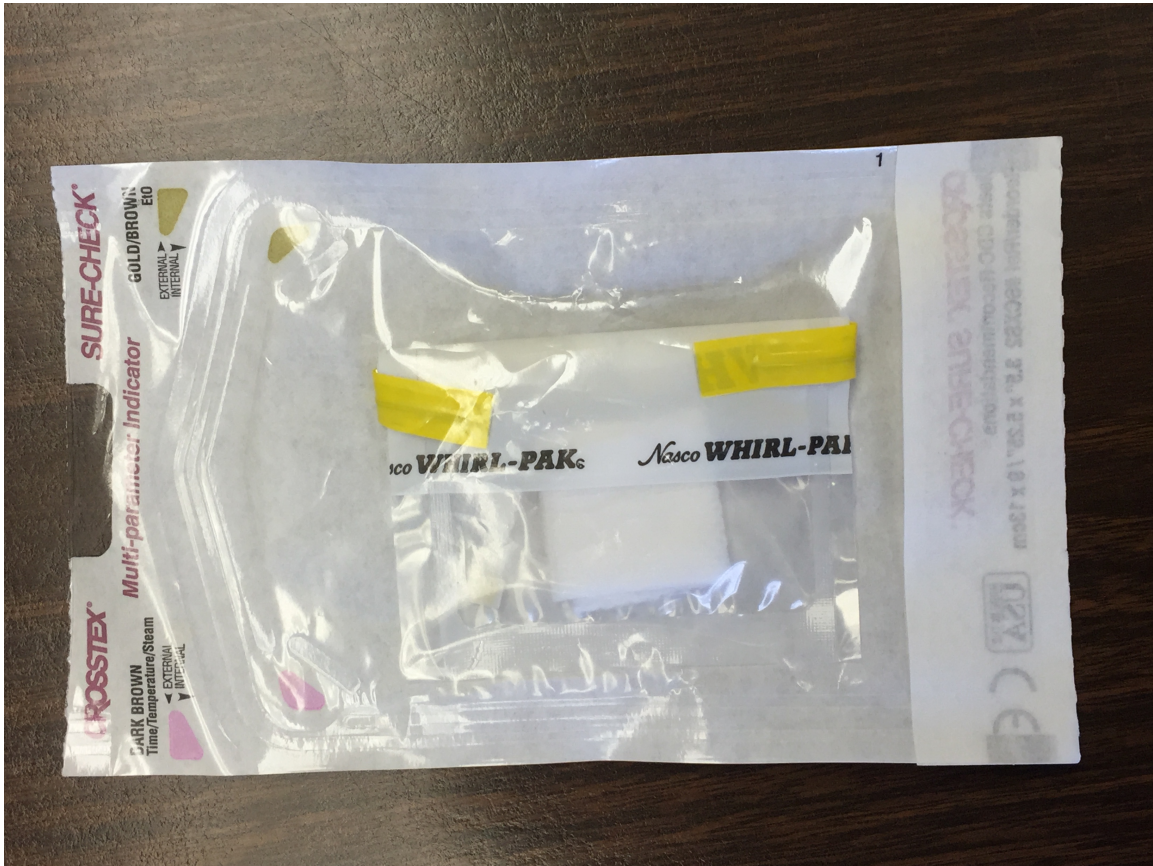


FIGURE 10	Dentin specimens were individually packaged and sterlized.
-----------	--



FIGURE 11. Work station where dentin specimens were transferred into sterile 96 well plates prior to antibiotic treatment.



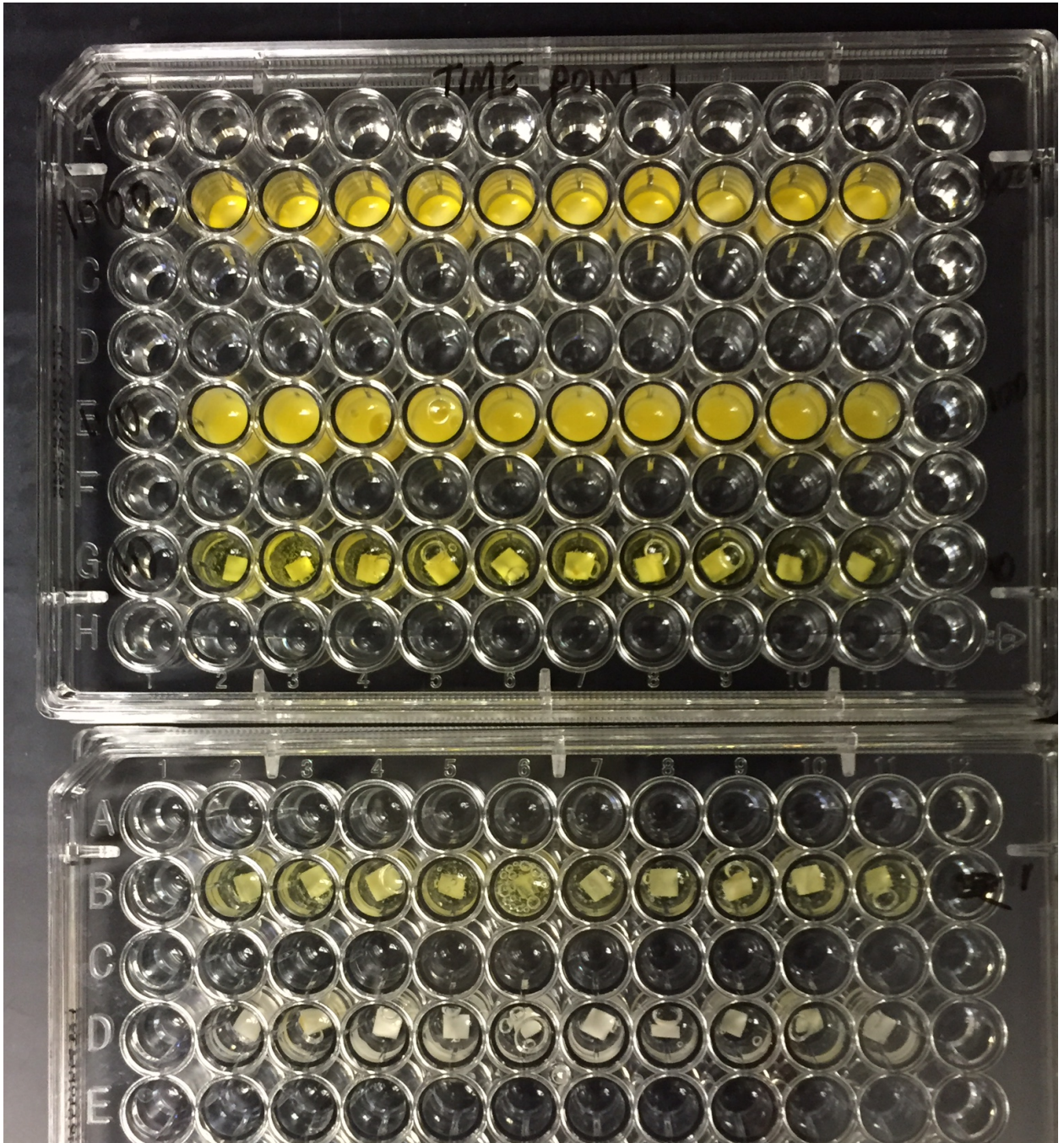


FIGURE 12. Dentin specimens placed in their perspective treatment groups. Starting from the top with group 1: 1000 mg/mL pure TAP; group 2: 100 mg/mL MC-TAP; group 3: 10 mg/mL MC-TAP; group 4: 1 mg/mL MC-TAP; group 5: MC only without TAP.



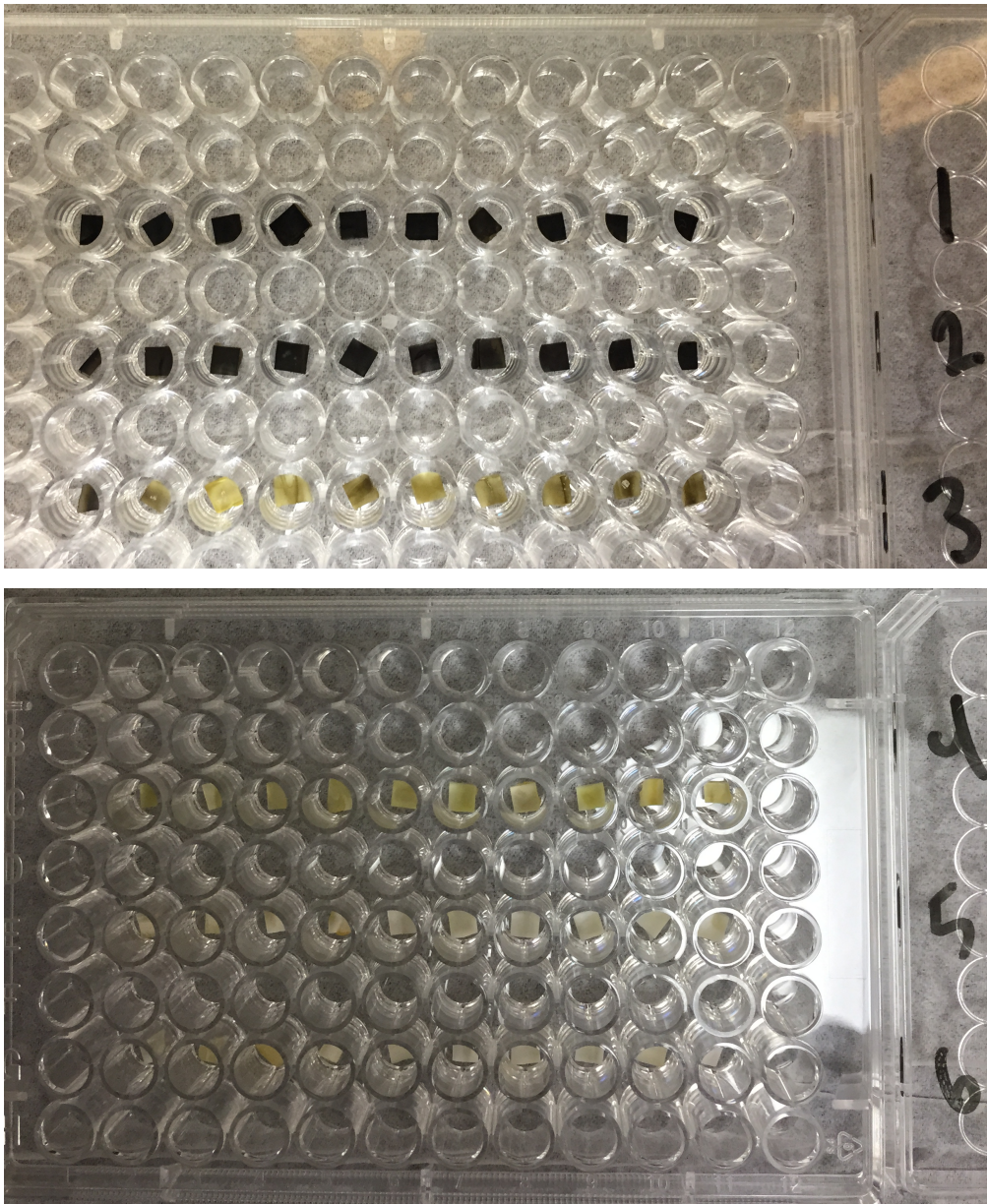


FIGURE 13. Dentin specimens after 3 weeks of treatment. Starting from the top with group 1: 1000 mg/mL pure TAP; group 2: 100 mg/mL MC-TAP; group 3: 10 mg/mL MC-TAP; group 4: 1 mg/mL MC-TAP; group 5: MC only without TAP; group 6: NaOCl.

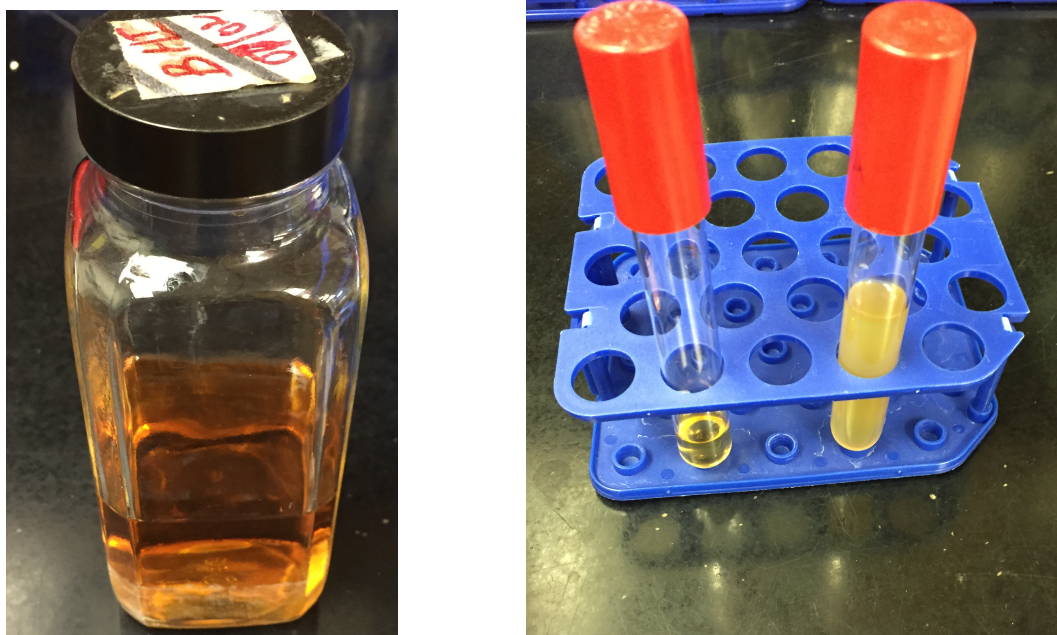


FIGURE 14. Preparation of *E. faecalis* inoculum with BHI media.



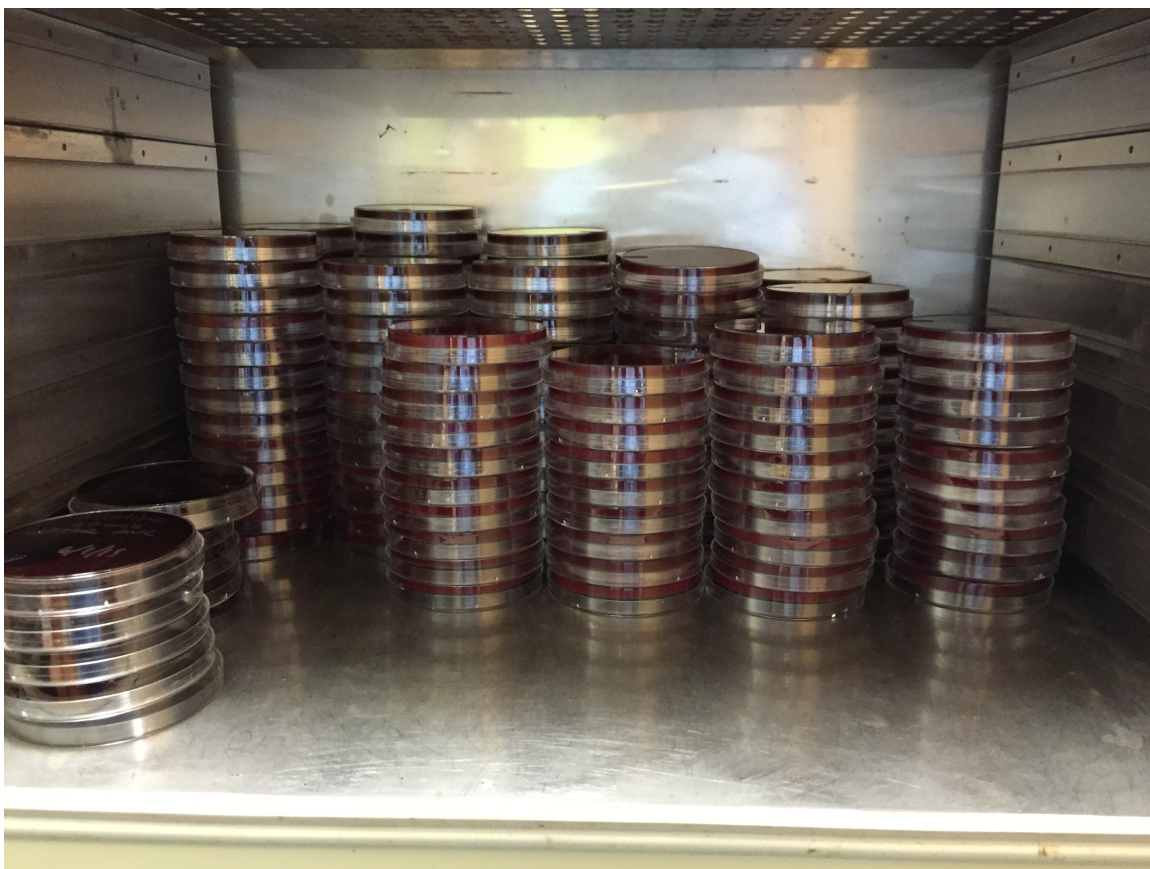
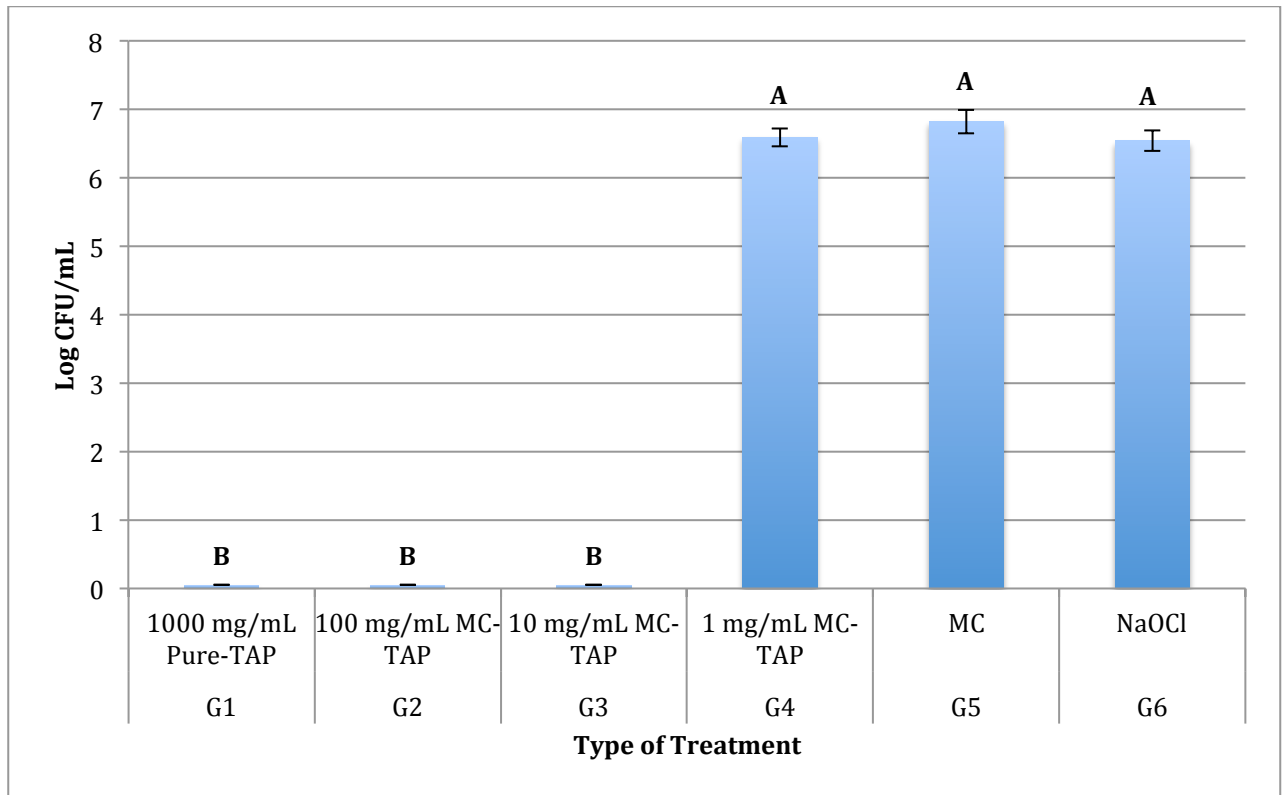


FIGURE 15.	Blood agar plates in incubator after spiral plating.
------------	--

GROUP	Material	2 weeks	SE	4 weeks	SE
1	1000 mg/mL	0	0	1.09	0.43
2	100 mg/mL	0	0	0	0
3	10 mg/mL	0	0	3.15	0.15
4	1 mg/mL	6.59	0.13	6.54	0.15
5	MC	6.82	0.17	7.24	0.07
6	1.5 % NaOCl	6.54	0.15	7.25	0.08

Table 1.	The mean and SE of log CFL/mL for bacterial biofilm grown for 3 weeks on dentin previously treated with different concentrations of TAP followed by immersion in PBS for 2 or 4 weeks.
----------	--



**FIGURE 16.** The mean  $\pm$  SE of log CFL/mL for bacterial biofilm grown for 3 weeks on dentin previously treated with different concentrations of TAP followed by immersion in PBS for 2 weeks. Different upper case letters represent statistical significance between different treatments.

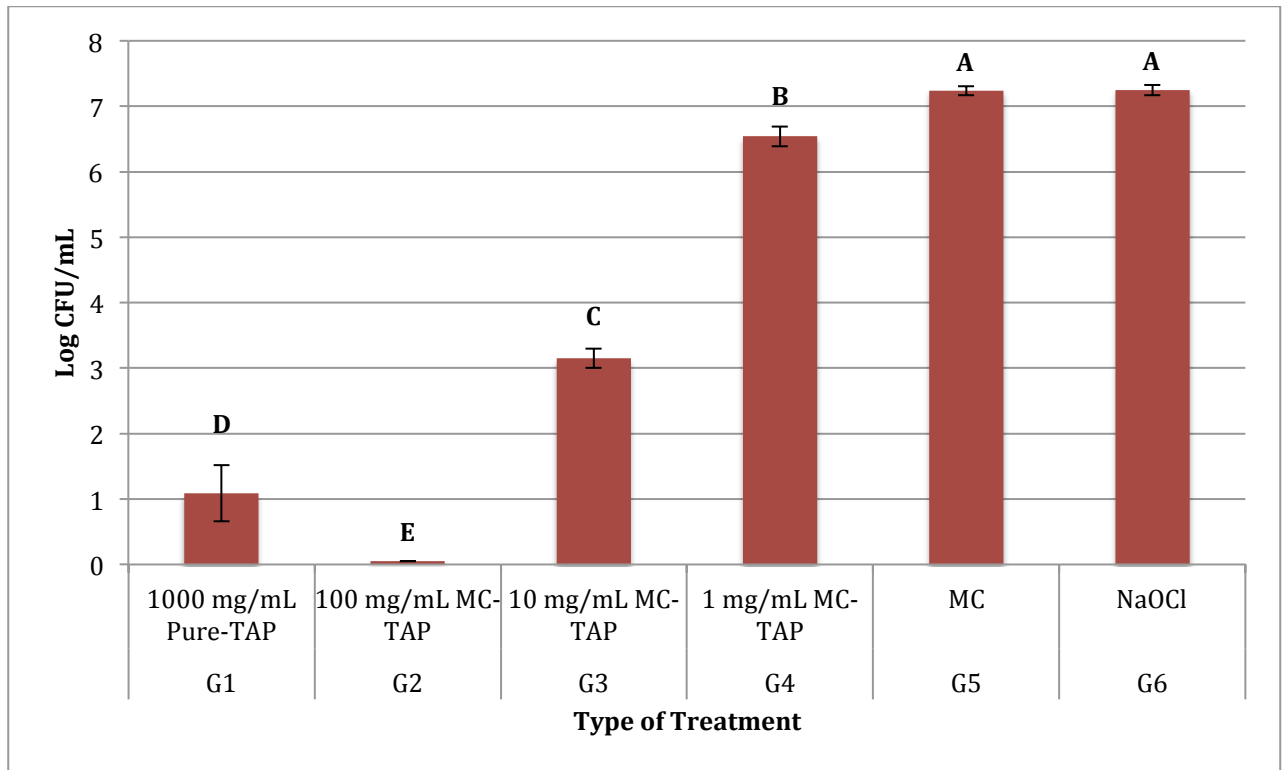
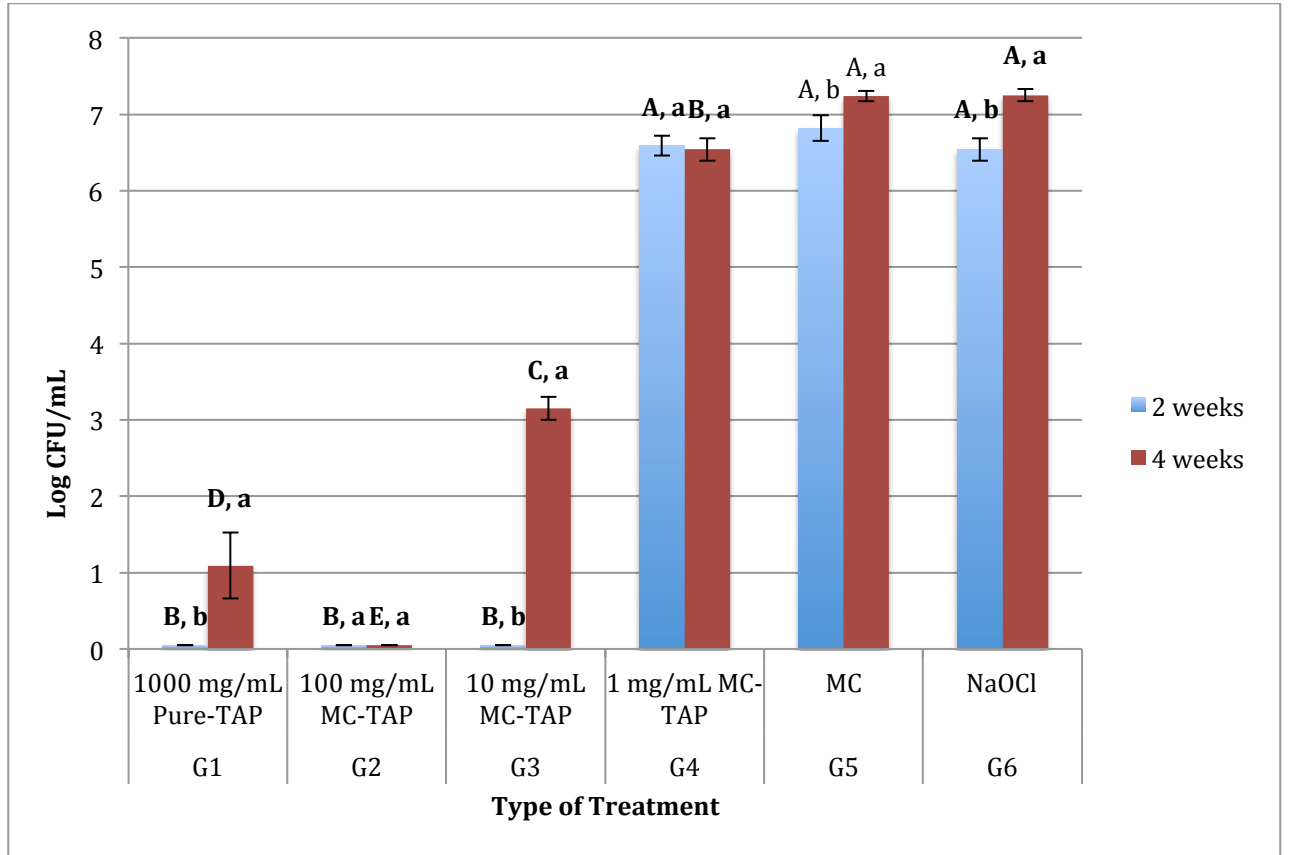


FIGURE 17	The mean $\pm$ SE of log CFL/mL for bacterial biofilm grown for 3 weeks on dentin previously treated with different concentrations of TAP followed by immersion in PBS for 4 weeks. Different upper case letters represent statistical significance between different treatments.
-----------	---



**FIGURE 18.** The mean  $\pm$  SE of log CFL/mL for bacterial biofilm grown for 3 weeks on dentin previously treated with different concentrations of TAP followed by immersion in PBS for 2 or 4 weeks. Different upper case letters represent statistical significance between different treatments within the same immersion time. Different lower case letters represent statistical significance between similar treatments within the two immersion times.



## **DISCUSSION**

TAP is the most commonly used medicament during regenerative endodontics. A relatively recent review of literature reported that TAP has been used in more than 50% of clinical endodontic regeneration cases published in the literature.<sup>202</sup> However, an excessive amount of TAP powder is usually required to mix with water in order to create a creamy consistency that can be clinically applied into the canal as an interappointment medicament (1000 mg/mL). This high concentration was suggested to have an unfavorable effect on SCAP<sup>203</sup> and DPSC,<sup>204</sup> as well as chemical,<sup>205</sup> physical,<sup>206</sup> and mechanical properties of dentin.<sup>177</sup> Therefore, the current study investigated the ability of creamy consistency of low concentrations of TAP loaded into a methylcellulose system to maintain the antibacterial properties of the original antibiotic mixture in an attempt to introduce a balanced disinfection protocol that can eliminate the infection without compromising the biological environment within the root canal system and the structural integrity of radicular dentin.

In the current study, dentin treated with 10 mg/mL of TAP or higher completely prevented the colonization of *E. faecalis* up to 5 weeks (2 weeks immersion in PBS plus 3 weeks period for biofilm colonization) after TAP removal. Additionally, dentin treated with 10 mg/mL of TAP or higher was able to exert a significant and substantial residual antibacterial effect (more than 4 log<sub>10</sub> reduction in CFU/mL) up to 7 weeks after TAP removal. On the other hand, dentin treated with 1 mg/mL of TAP did not demonstrate a substantial residual antibacterial effect. A recent study suggested that dentin treated with 1 mg/mL of TAP solution exerted a residual antibacterial effect up to 2 weeks after TAP removal.<sup>207</sup> The disagreement between the current study and the previous one.<sup>207</sup> In regard to residual antibacterial effect of dentin treated with 1 mg/mL of TAP could be

explained by the longer time interval between removing the medicament and investigating the residual antibacterial effects, which was 5-7 weeks in the current study and only 2 weeks in the previous study. The disagreement could be also explained by the use of mature 3-week old biofilm in the current study in comparison to the use of 3-day young biofilm in the previous study. It is also worth noting that EDTA was used as a final irrigation step after TAP removal in the current study as an attempt to replicate the actual clinical scenario during endodontic regeneration.

A recent study was performed at IUSD at the same time as this study to investigate the direct effect of a low concentration of TAP loaded into the methylcellulose system. With a protocol similar to the one described in this study, 60 dentin samples were inoculated with *E. faecalis* and incubated for 3 weeks. These samples were then randomized into the same 6 treatment groups (n=10) of 1000 mg/mL of pure TAP, 100, 10, 1 mg/mL MC-TAP, MC placebo paste, and 5 minutes of 1.5% NaOCl. The samples were incubated for 3 weeks and they were placed in a biofilm disruption assay to measure the direct effect of MC-TAP. The results indicated that a 3 week application of all tested concentrations of TAP as well as the 5 minutes of application of 1.5% NaOCl were able to completely eradicate an established *E. faecalis* biofilm in comparison to the placebo paste. This suggests that a concentration as low as 1 mg/mL was successfully loaded into the aqueous methylcellulose system and can efficiently be used as an intracanal medicament during endodontic regeneration. These findings are in agreement with a recent published study that suggested direct antibacterial effect of 1 mg/mL of TAP in a liquid form against established *E. faecalis* biofilm.<sup>208</sup> Furthermore, 1 mg/mL of TAP solution was suggested to have no cytotoxic effect against

stem cells from apical papillae.<sup>203</sup> Additionally, 1 mg/mL of TAP loaded into a methylcellulose system was found to have minimum negative effects on the structural integrity and mechanical properties of dentin in comparison to the clinically used concentration of TAP.<sup>209</sup> Taking all into consideration, it seems like 1 mg/mL TAP is able to have a significant direct antibacterial effect against an established biofilm but was unable to exert an extended residual antibacterial effect.

The residual antibacterial effect of dentin treated with TAP reported in the current study could be explained by the ability of TAP to bind to dentin and gradually released in active form. The minimum bactericidal concentration of TAP against *E. faecalis* was found to be 0.3 mg/mL.<sup>210</sup> Therefore, it is expected that dentin previously treated with TAP should be able to release at least 0.3 mg/mL of TAP to maintain a substantial antibacterial effect at specific time point. A recent study suggested that 85% of radiolabeled TAP was retained within radicular dentin after various irrigation methods.<sup>211</sup> It is well documented that various tetracycline derivatives have the ability to bind to collagen and exert residual antibacterial effects.<sup>212, 213</sup> Consequently, the presence of minocycline in TAP might be helpful in explaining the residual antibacterial properties of dentin previously treated with TAP. However, a recent study demonstrated that dentin treated with a minocycline free antibiotic combination, namely double antibiotic paste (equal portions of ciprofloxacin and metronidazole) can also exert residual antibacterial effects.<sup>207</sup> Therefore, it may be logical to assume that more than a single antibiotic component in TAP can be helpful in charging the dentin with extended antibacterial properties.

For experimental groups examined 7 weeks after TAP removal, dentin treated with 100 mg/mL of methylcellulose-based TAP demonstrated a significantly higher residual antibacterial effect in comparison to dentin treated with 1000 mg/mL of TAP (clinically used concentration prepared without methylcellulose). This indicates that the use of the methylcellulose system may be helpful in extending the antibacterial properties of TAP in the oral environment by slowing down the antibiotic degradation process and/or improving the retention of TAP within radicular dentin. Previous studies have also demonstrated that the incorporation of  $\text{Ca}(\text{OH})_2$  into an aqueous methylcellulose system caused significantly better antibacterial effects against various endodontic pathogens in comparison to higher concentrations of  $\text{Ca}(\text{OH})_2$  pastes prepared without methylcellulose.<sup>214, 215</sup> It is also worth noting that methylcellulose is frequently used as culture media for stem cell growth and differentiation due to its non-cytotoxic nature.<sup>216</sup> Indeed, various concentrations of aqueous methylcellulose paste were proposed to improve the proliferation of dental pulp stem cells in a recent study.<sup>217</sup> Therefore, methylcellulose loaded with specific low concentrations of antibiotics may be deliberately used as a stem cell friendly interappointment antimicrobial medicament during endodontic regeneration. However, future studies are warranted to investigate the cytotoxic nature of various concentrations of TAP loaded into an aqueous methylcellulose system.

Previous studies have shown that 5 minutes of irrigation with 1-1.5% NaOCl caused elimination of bacterial biofilm<sup>218, 219</sup>. However, dentin treated with 1.5% of NaOCl did not induce significant residual antibacterial effects in the current study. Previous studies have also suggested limited residual antibacterial properties of

NaOCl.<sup>100, 212</sup> Few endodontic regeneration case reports have demonstrated successful root canal disinfection using irrigation solutions without supplementary interappointment medicaments.<sup>220, 221</sup> However, the use of antibiotic intracanal medicaments such as TAP in addition to irrigation solutions can be extremely helpful when an extended residual antibacterial effect is required.<sup>222</sup> This may be essential in endodontic regeneration cases with established bacterial infections that are clinically presented with necrotic pulp, acute or chronic abscess, and/or a radiographically visible periapical lesion.<sup>223</sup>

One of the most cited disadvantages of TAP is the associated discoloration of the treated teeth, which has been shown through various clinical and *in vitro* studies.<sup>156, 182, 224</sup> This is especially problematic in treating anterior teeth that are in the esthetic zone. Studies have attributed the discoloration to the presence of minocycline, or to a lesser degree, the presence of any tetracycline derivatives.<sup>225</sup> It is suggested that the minocycline binds to the calcium within the dentin, hence causing a chelation reaction that results in staining the substrate.<sup>225</sup> Many efforts were considered to reduce the discoloration associated with TAP treatments. These include applying a bonding agent to the dentin tubules prior to TAP treatment,<sup>182</sup> utilizing a metal tip to deliver the TAP below the coronal aspect of the tooth,<sup>226</sup> altering the composition of TAP by substituting the minocycline with a different antibiotic like amoxicillin,<sup>227</sup> and utilizing electro-spun scaffolds loaded with TAP.<sup>224</sup> In the current study, discoloration was not part of the experimental protocol; however, some observations were noted. Methylcellulose did not cause any staining on the dentin samples when compared to the control untreated samples. Staining was observed on all dentin samples treated with the different concentrations of TAP; however, the effect was concentration dependent. Samples treated

with 1000 mg/mL of TAP showed a greater degree of discoloration compared to those treated with 1 mg/mL of TAP. It is important to note that the discoloration observed in the current study was as a result of direct application of TAP on thin dentin samples and it might not correlate clinically as it may not reach the crown or cementum due to the incorporation of the TAP into the methylcellulose. A study is currently being developed to closely examine the staining effect and the level of dentin penetration especially with low concentrations of TAP loaded into methylcellulose gel.

## **SUMMARY AND CONCLUSIONS**



Our hypothesis stating that radicular dentin treated with various dilutions of MC-based TAP will have no significant residual antibacterial effect on both tested time points was rejected. The current study demonstrated the ability of low concentrations of TAP loaded into a methylcellulose system to maintain their antibacterial properties in comparison to the clinically used higher concentration (1000 mg/mL). Furthermore, at least 10 mg/mL of methylcellulose-based TAP should be used to obtain extended residual antibacterial effects. Further studies are warranted to investigate the cytotoxic potentials of various concentrations of methylcellulose based TAP.

## REFERENCES

1. Schour I MM. Studies in tooth development: the growth pattern of human teeth, part II Journal of American Dental Association 1940(27, ):1918-1931.
2. Gomes-Filho JE DP, de Oliveira CB, Watanabe S, Lodi CS, Cintra LT et al. Tissue reaction to a triantibiotic paste used for endodontic tissue self-regeneration of nonvital immature permanent teeth. Journal of Endodontics 2012;38:91-94.
3. Cvek M. Prognosis of luxated non-vital maxillary incisors treated with calcium hydroxide and filled with gutta-percha. A retrospective clinical study. Endod Dent Traumatol 1992;8(2):45-55.
4. Fusayama T, Maeda T. Effect of pulpectomy on dentin hardness. J Dent Res 1969;48(3):452-460.
5. Steiner JC, Van Hassel HJ. Experimental root apexification in primates. Oral Surg Oral Med Oral Pathol 1971;31(3):409-415.
6. Steiner JC, Dow PR, Cathey GM. Inducing root end closure of nonvital permanent teeth. J Dent Child 1968;35(1):47-54.
7. Frank AL. Therapy for the divergent pulpless tooth by continued apical formation. J Am Dent Assoc 1966;72(1):87-93.
8. Andreasen JO, Farik B, Munksgaard EC. Long-term calcium hydroxide as a root canal dressing may increase risk of root fracture. Dental Traumatology 2002;18(3):134-137.
9. Trope M. Treatment of the immature tooth with a non-vital pulp and apical periodontitis. Dent Clin North Am 2010;54(2):313-324.
10. Bose R, Nummikoski P, Hargreaves K. A retrospective evaluation of radiographic outcomes in immature teeth with necrotic root canal systems treated with regenerative endodontic procedures. J Endod 2009;35(10):1343-1349.
11. Jeeruphan T, Jantararat J, Yanpiset K, Suwannapan L, Khewsawai P, Hargreaves KM. Mahidol study 1: comparison of radiographic and survival outcomes of immature teeth treated with either regenerative endodontic or apexification methods: a retrospective study. J Endod 2012;38(10):1330-1336.
12. Endodontists AAo. Considerations for Regenerative Procedures. 2013 [cited 2013 February 4, 2013]; Available from:
13. Nakashima M, Akamine A. The application of tissue engineering to regeneration of pulp and dentin in endodontics. J Endod 2005;31(10):711-718.

14. Lovelace TW, Henry MA, Hargreaves KM, Diogenes A. Evaluation of the delivery of mesenchymal stem cells into the root canal space of necrotic immature teeth after clinical regenerative endodontic procedure. *J Endod* 2011;37(2):133-138.
15. Galler KM, D'Souza RN, Federlin M, Cavender AC, Hartgerink JD, Hecker S, et al. Dentin conditioning codetermines cell fate in regenerative endodontics. *J Endod* 2011;37(11):1536-1541.
16. Diogenes A HMA, Teixeira FB, & Hargreaves KM. An update on clinical regenerative endodontics. *ENDODONTIC TOPICS* 2013;28:2-23.
17. Verdelis K, Eliades G, Oviir T, Margelos J. Effect of chelating agents on the molecular composition and extent of decalcification at cervical, middle and apical root dentin locations. *Endod Dent Traumatol* 1999;15(4):164-170.
18. Fukuzaki S. Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. *Biocontrol Sci* 2006;11(4):147-157.
19. Essner MD, Javed A, Eleazer PD. Effect of sodium hypochlorite on human pulp cells: an in vitro study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2011;112(5):662-666.
20. Yassen GH, Al-Angari SS, Platt JA. The use of traditional and novel techniques to determine the hardness and indentation properties of immature radicular dentin treated with antibiotic medicaments followed by ethylenediaminetetraacetic acid. *Eur J Dent* 2014;8(4):521-527.
21. Sabrah AH, Yassen GH, Gregory RL. Effectiveness of antibiotic medicaments against biofilm formation of *Enterococcus faecalis* and *Porphyromonas gingivalis*. *J Endod* 2013;39(11):1385-1389.
22. Ings RM MJ, Ormerod WE. The mode of action of metronidazole in *Trichomonas vaginalis* and other micro-organisms. *Biochemical Pharmacology* 1974;23:1421-1429.
23. JA. B. The antimicrobial armamentarium: evaluating current and future treatment options. *Pharmacotherapy* 2005;25:55S-62S.
24. Fouad AF, Verma P. Healing after regenerative procedures with and without pulpal infection. *J Endod* 2014;40(4 Suppl):S58-64.
25. Fouad AF. The microbial challenge to pulp regeneration. *Adv Dent Res* 2011;23(3):285-289.
26. Nerness AZ, Ehrlich Y, Spolnik K, Platt JA, Yassen GH. Effect of triple antibiotic paste with or without ethylenediaminetetraacetic acid on surface loss and surface roughness of radicular dentine. *Odontology* 2015.

27. Labban N, Yassen GH, Windsor LJ, Platt JA. The direct cytotoxic effects of medicaments used in endodontic regeneration on human dental pulp cells. *Dent Traumatol* 2014;30(6):429-434.
28. Chuensombat S, Khemaleelakul S, Chattipakorn S, Srisuwan T. Cytotoxic effects and antibacterial efficacy of a 3-antibiotic combination: an in vitro study. *J Endod* 2013;39(6):813-819.
29. Ruparel NB, Teixeira FB, Ferraz CC, Diogenes A. Direct effect of intracanal medicaments on survival of stem cells of the apical papilla. *J Endod* 2012;38(10):1372-1375.
30. Althumairy RI, Teixeira FB, Diogenes A. Effect of dentin conditioning with intracanal medicaments on survival of stem cells of apical papilla. *J Endod* 2014;40(4):521-525.
31. Hoshino E, Kurihara-Ando N, Sato I, Uematsu H, Sato M, Kota K, et al. In-vitro antibacterial susceptibility of bacteria taken from infected root dentine to a mixture of ciprofloxacin, metronidazole and minocycline. *Int Endod J* 1996;29(2):125-130.
32. Sabrah AH, Yassen GH, Liu WC, Goebel WS, Gregory RL, Platt JA. The effect of diluted triple and double antibiotic pastes on dental pulp stem cells and established *Enterococcus faecalis* biofilm. *Clin Oral Investig* 2015.
33. P. J. Baker RTE, R. A. Coburn and R. J. Genco. Tetracycline and Its Derivatives Strongly Bind to and Are Released From the Tooth Surface in Active Form. *Journal of Periodontology* 1982;54(10):580-585.
34. Sabrah AH. Diluted antibiotics for treating traumatized immature teeth [Dissertation]. Indiana University School of Dentistry; 2014.
35. Bowles WH BT. Staining of adult teeth by minocycline: binding of minocycline by specific proteins. *Journal of Esthetic Dentistry* 1997;9(30-4).
36. Mohammadi Z GL, Mombeinipour A. Antibacterial substantivity of a new antibiotic-based endodontic irrigation solution. *Australian Endodontic Journal* 2012;38:26-30.
37. Prather B, Ehrlich Y, Spolnik K, Platt J, Yassen G. Effects of two combinations of triple antibiotic paste used in endodontic regeneration on root microhardness and chemical structure of radicular dentine. *Journal of Oral Science* 2014;56(4):245-251.
38. Lambrianidis T, Margelos J, Beltes P. Removal efficiency of calcium hydroxide dressing from the root canal. *Journal of Endodontics* 1999;25(2):85-88.

39. Cruse WP, Bellizzi R. A historic review of endodontics, 1689-1963, part 1. *J Endod* 1980;6(3):495-499.
40. Curson I. History and Endodontics. *Dent Pract Dent Rec* 1965;15:435-439.
41. Francke OC. Capping of the living pulp: from Philip Pfaff to John Wessler. *Bull Hist Dent* 1971;19(2):17-23.
42. Farley JR. Brief history of endodontics. *Tex Dent J* 1974;92(2):9.
43. Koch CRECRET, Burton Lee. History of dental surgery. Ft. Wayne, Ind. : National Art Pub. Co.; 1910.
44. G D. The history of vitalism in pulp treatment. *Dent Cosmos* 1931;73:267-273.
45. Lightfoot J. A brief history of root canal therapy. *Dent Student* 1955;33(42):11-17.
46. WS. M. Outline of dental history. Fairleigh Dickinson University Dental School; 1972.
47. Grossman LPALI. A Brief History of Root-Canal Therapy in the United States. *J Am Dent Assoc* 1945;32(1):43-50.
48. Grossman LI. Endodontics: a peep into the past and the future. *Oral Surg Oral Med Oral Pathol* 1974;37(4):599-608.
49. Ostrander FD. The practice of endodontics: past, present, and future. *J Dent Educ* 1967;31(3):386-388.
50. Tagger M. Endodontics: a review of the past and its present status. *Alpha Omegan* 1967;60(2):107-118.
51. Cruse WP, Bellizzi R. A historic review of endodontics, 1689-1963, part 2. *J Endod* 1980;6(4):532-535.
52. Bellizzi R, Cruse WP. A historic review of endodontics, 1689-1963, part 3. *J Endod* 1980;6(5):576-580.
53. Jacobsohn PH, Fedran RJ. Making darkness visible: the discovery of X-ray and its introduction to dentistry. *J Am Dent Assoc* 1995;126(10):1359-1367.
54. Coolidge ED. Past and present concepts in endodontics. *J Am Dent Assoc* 1960;61:676-688.

55. Herschfeld JJ. William Hunter and the role of "oral sepsis" in American dentistry. *Bull Hist Dent* 1985;33(1):35-45.
56. Pallasch TJ, Wahl MJ. The focal infection theory: appraisal and reappraisal. *J Calif Dent Assoc* 2000;28(3):194-200.
57. Dussault G, Sheiham A. Medical theories and professional development. The theory of focal sepsis and dentistry in early twentieth century Britain. *Soc Sci Med* 1982;16(15):1405-1412.
58. Glick DH. Endodontics: past, present and future. *Alpha Omegan* 1968;61(2):124-126.
59. Grossman LI. Endodontics 1776-1976: a bicentennial history against the background of general dentistry. *J Am Dent Assoc* 1976;93(1):78-87.
60. Karapinar-Kazandag M, Basrani BR, Friedman S. The operating microscope enhances detection and negotiation of accessory mesial canals in mandibular molars. *J Endod* 2010;36(8):1289-1294.
61. Kim S, Kratchman S. Modern endodontic surgery concepts and practice: a review. *J Endod* 2006;32(7):601-623.
62. Torabinejad M, Hong CU, McDonald F, Pitt Ford TR. Physical and chemical properties of a new root-end filling material. *J Endod* 1995;21(7):349-353.
63. Patel S, Dawood A, Wilson R, Horner K, Mannocci F. The detection and management of root resorption lesions using intraoral radiography and cone beam computed tomography - an in vivo investigation. *Int Endod J* 2009;42(9):831-838.
64. Abella F, Patel S, Duran-Sindreu F, Mercade M, Bueno R, Roig M. Evaluating the periapical status of teeth with irreversible pulpitis by using cone-beam computed tomography scanning and periapical radiographs. *J Endod* 2012;38(12):1588-1591.
65. Setzer FC, Shah SB, Kohli MR, Karabucak B, Kim S. Outcome of endodontic surgery: a meta-analysis of the literature--part 1: Comparison of traditional root-end surgery and endodontic microsurgery. *J Endod* 2010;36(11):1757-1765.
66. Gutmann J. AAE History: 1990-2011. .
67. Glickman G. Becoming the specialty that cares. *J Endod* 2000;36:169.
68. AAE. Scope of Endodontics: Regenerative Endodontics. *J Endod* 2013;39:561.

69. Kakehashi S, Stanley HR, Fitzgerald RJ. The Effects of Surgical Exposures of Dental Pulps in Germ-Free and Conventional Laboratory Rats. *Oral Surg Oral Med Oral Pathol* 1965;20:340-349.
70. Moller AJ, Fabricius L, Dahlen G, Ohman AE, Heyden G. Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. *Scand J Dent Res* 1981;89(6):475-484.
71. Schilder H. Filling root canals in three dimensions. *Dent Clin North Am* 1967;723-744.
72. Schilder H. Cleaning and shaping the root canal. *Dent Clin North Am* 1974;18(2):269-296.
73. Siqueira JF, Jr. Aetiology of root canal treatment failure: why well-treated teeth can fail. *Int Endod J* 2001;34(1):1-10.
74. OA Peters CP. *Pathway of the pulp*. St Louis: Mosby Inc; 2006.
75. Hulsmann M, Rummelin C, Schaefers F. Root canal cleanliness after preparation with different endodontic handpieces and hand instruments: a comparative SEM investigation. *J Endod* 1997;23(5):301-306.
76. Abbott PV. The periapical space--a dynamic interface. *Aust Endod J* 2002;28(3):96-107.
77. Stewart GG. The importance of chemomechanical preparation of the root canal. *Oral Surg Oral Med Oral Pathol* 1955;8(9):993-997.
78. Grossman L. Rationale of endodontic treatment. *Dent Clin North Am* 1967;483-490.
79. Heuer MA. [Endodontic Therapy (Biomechanic Preparation)]. *Dent Cadmos* 1965;33:17-18 PASSIM.
80. Khademi A, Yazdizadeh M, Feizianfard M. Determination of the minimum instrumentation size for penetration of irrigants to the apical third of root canal systems. *J Endod* 2006;32(5):417-420.
81. Pettiette MT, Delano EO, Trope M. Evaluation of success rate of endodontic treatment performed by students with stainless-steel K-files and nickel-titanium hand files. *J Endod* 2001;27(2):124-127.
82. Peters OA, Schonenberger K, Laib A. Effects of four Ni-Ti preparation techniques on root canal geometry assessed by micro computed tomography. *Int Endod J* 2001;34(3):221-230.



83. Peters OA, Laib A, Gohring TN, Barbakow F. Changes in root canal geometry after preparation assessed by high-resolution computed tomography. *J Endod* 2001;27(1):1-6.
84. Hubscher W, Barbakow F, Peters OA. Root-canal preparation with FlexMaster: canal shapes analysed by micro-computed tomography. *Int Endod J* 2003;36(11):740-747.
85. De Deus QD. Frequency, location, and direction of the lateral, secondary, and accessory canals. *J Endod* 1975;1(11):361-366.
86. Love RM, Jenkinson HF. Invasion of dentinal tubules by oral bacteria. *Crit Rev Oral Biol Med* 2002;13(2):171-183.
87. Zou L, Shen Y, Li W, Haapasalo M. Penetration of sodium hypochlorite into dentin. *J Endod* 2010;36(5):793-796.
88. Zehnder M. Root canal irrigants. *J Endod* 2006;32(5):389-398.
89. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev* 1999;12(1):147-179.
90. Barrette WC, Jr., Hannum DM, Wheeler WD, Hurst JK. General mechanism for the bacterial toxicity of hypochlorous acid: abolition of ATP production. *Biochemistry* 1989;28(23):9172-9178.
91. McKenna SM, Davies KJ. The inhibition of bacterial growth by hypochlorous acid. Possible role in the bactericidal activity of phagocytes. *Biochem J* 1988;254(3):685-692.
92. Hand RE, Smith ML, Harrison JW. Analysis of the effect of dilution on the necrotic tissue dissolution property of sodium hypochlorite. *J Endod* 1978;4(2):60-64.
93. Moorer WR, Wesselink PR. Factors promoting the tissue dissolving capability of sodium hypochlorite. *Int Endod J* 1982;15(4):187-196.
94. McComb D, Smith DC. A preliminary scanning electron microscopic study of root canals after endodontic procedures. *J Endod* 1975;1(7):238-242.
95. Torabinejad M, Handysides R, Khademi AA, Bakland LK. Clinical implications of the smear layer in endodontics: a review. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002;94(6):658-666.
96. Calt S, Serper A. Time-dependent effects of EDTA on dentin structures. *J Endod* 2002;28(1):17-19.

97. Niu W, Yoshioka T, Kobayashi C, Suda H. A scanning electron microscopic study of dentinal erosion by final irrigation with EDTA and NaOCl solutions. *Int Endod J* 2002;35(11):934-939.
98. Shahravan A, Haghdoost AA, Adl A, Rahimi H, Shadifar F. Effect of smear layer on sealing ability of canal obturation: a systematic review and meta-analysis. *J Endod* 2007;33(2):96-105.
99. Martinho FC, Gomes BP. Quantification of endotoxins and cultivable bacteria in root canal infection before and after chemomechanical preparation with 2.5% sodium hypochlorite. *J Endod* 2008;34(3):268-272.
100. Weber CD, McClanahan SB, Miller GA, Diener-West M, Johnson JD. The effect of passive ultrasonic activation of 2% chlorhexidine or 5.25% sodium hypochlorite irrigant on residual antimicrobial activity in root canals. *J Endod* 2003;29(9):562-564.
101. Baca P, Mendoza-Llamas ML, Arias-Moliz MT, Gonzalez-Rodriguez MP, Ferrer-Luque CM. Residual effectiveness of final irrigation regimens on *Enterococcus faecalis*-infected root canals. *J Endod* 2011;37(8):1121-1123.
102. Ohara P, Torabinejad M, Kettering JD. Antibacterial effects of various endodontic medicaments on selected anaerobic bacteria. *J Endod* 1993;19(10):498-500.
103. Komorowski R, Grad H, Wu XY, Friedman S. Antimicrobial substantivity of chlorhexidine-treated bovine root dentin. *J Endod* 2000;26(6):315-317.
104. Hennessey TS. Some antibacterial properties of chlorhexidine. *J Periodontal Res Suppl* 1973;12:61-67.
105. Hugo WB, Longworth AR. The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constituents, dehydrogenase activity and cell walls of *Escherichia coli* and *Staphylococcus aureus*. *J Pharm Pharmacol* 1966;18(9):569-578.
106. Basrani BR, Manek S, Mathers D, Fillery E, Sodhi RN. Determination of 4-chloroaniline and its derivatives formed in the interaction of sodium hypochlorite and chlorhexidine by using gas chromatography. *J Endod* 2010;36(2):312-314.
107. Nowicki JB, Sem DS. An in vitro spectroscopic analysis to determine the chemical composition of the precipitate formed by mixing sodium hypochlorite and chlorhexidine. *J Endod* 2011;37(7):983-988.
108. Schaeffer MA, White RR, Walton RE. Determining the optimal obturation length: a meta-analysis of literature. *J Endod* 2005;31(4):271-274.

109. Ng YL, Mann V, Gulabivala K. A prospective study of the factors affecting outcomes of non-surgical root canal treatment: part 2: tooth survival. *Int Endod J* 2011;44(7):610-625.
110. Berman L. *Cohen's Pathways of the pulp*. 10 ed. St Louis: Mosby Inc; 2011.
111. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1998;85(1):86-93.
112. Nagata JY, Soares AJ, Souza-Filho FJ, Zaia AA, Ferraz CC, Almeida JF, et al. Microbial evaluation of traumatized teeth treated with triple antibiotic paste or calcium hydroxide with 2% chlorhexidine gel in pulp revascularization. *J Endod* 2014;40(6):778-783.
113. Brennan MJ, Cisar JO, Vatter AE, Sandberg AL. Lectin-dependent attachment of *Actinomyces naeslundii* to receptors on epithelial cells. *Infect Immun* 1984;46(2):459-464.
114. Liljemark WF, Bloomquist CG, Bandt CL, Pihlstrom BL, Hinrichs JE, Wolff LF. Comparison of the distribution of *Actinomyces* in dental plaque on inserted enamel and natural tooth surfaces in periodontal health and disease. *Oral Microbiol Immunol* 1993;8(1):5-15.
115. Wilson M, Reddi K, Henderson B. Cytokine-inducing components of periodontopathogenic bacteria. *J Periodontal Res* 1996;31(6):393-407.
116. Hotokezaka H, Sakai E, Ohara N, Hotokezaka Y, Gonzales C, Matsuo K, et al. Molecular analysis of RANKL-independent cell fusion of osteoclast-like cells induced by TNF- $\alpha$ , lipopolysaccharide, or peptidoglycan. *J Cell Biochem* 2007;101(1):122-134.
117. Reed MJ. Chemical and antigenic properties of the cell wall of *Actinomyces viscosus* (Strain T6). *J Dent Res* 1972;51(5):1193-1202.
118. Kumar A, Zhang J, Yu FS. Innate immune response of corneal epithelial cells to *Staphylococcus aureus* infection: role of peptidoglycan in stimulating proinflammatory cytokine secretion. *Invest Ophthalmol Vis Sci* 2004;45(10):3513-3522.
119. Bolstad AI, Jensen HB, Bakken V. Taxonomy, biology, and periodontal aspects of *Fusobacterium nucleatum*. *Clin Microbiol Rev* 1996;9(1):55-71.
120. Siqueira J. *Treatment of Endodontic Infections*. 1 ed; 2011.
121. Siqueira JF, Jr. Endodontic infections: concepts, paradigms, and perspectives. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002;94(3):281-293.

122. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. *Periodontol* 2000 1999;20:168-238.
123. Barnard D, Davies J, Figdor D. Susceptibility of *Actinomyces israelii* to antibiotics, sodium hypochlorite and calcium hydroxide. *Int Endod J* 1996;29(5):320-326.
124. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. *Crit Rev Oral Biol Med* 2004;15(6):348-381.
125. Sedgley CM, Lennan SL, Clewell DB. Prevalence, phenotype and genotype of oral enterococci. *Oral Microbiol Immunol* 2004;19(2):95-101.
126. Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. *Int Endod J* 2002;35(3):221-228.
127. Rocas IN, Siqueira JF, Jr., Santos KR. Association of *Enterococcus faecalis* with different forms of periradicular diseases. *J Endod* 2004;30(5):315-320.
128. Distel JW, Hatton JF, Gillespie MJ. Biofilm formation in medicated root canals. *J Endod* 2002;28(10):689-693.
129. Salehrabi R, Rotstein I. Endodontic treatment outcomes in a large patient population in the USA: an epidemiological study. *J Endod* 2004;30(12):846-850.
130. Ham JW, Patterson SS, Mitchell DF. Induced apical closure of immature pulpless teeth in monkeys. *Oral Surg Oral Med Oral Pathol* 1972;33(3):438-449.
131. Hargreaves K CS. *Pathways of the Pulp*. 10th ed. St Louis: Mosby. Inc; 2011.
132. Rosenberg B, Murray PE, Namerow K. The effect of calcium hydroxide root filling on dentin fracture strength. *Dent Traumatol* 2007;23(1):26-29.
133. Yassen GH, Platt JA. The effect of nonsetting calcium hydroxide on root fracture and mechanical properties of radicular dentine: a systematic review. *Int Endod J* 2013;46(2):112-118.
134. Doyon GE, Dumsha T, von Fraunhofer JA. Fracture resistance of human root dentin exposed to intracanal calcium hydroxide. *J Endod* 2005;31(12):895-897.
135. Valois CR, Costa ED, Jr. Influence of the thickness of mineral trioxide aggregate on sealing ability of root-end fillings in vitro. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2004;97(1):108-111.

136. Witherspoon DE, Ham K. One-visit apexification: technique for inducing root-end barrier formation in apical closures. *Pract Proced Aesthet Dent* 2001;13(6):455-460; quiz 462.
137. Holden DT, Schwartz SA, Kirkpatrick TC, Schindler WG. Clinical outcomes of artificial root-end barriers with mineral trioxide aggregate in teeth with immature apices. *J Endod* 2008;34(7):812-817.
138. Langer R, Vacanti JP. Tissue engineering. *Science* 1993;260(5110):920-926.
139. A N. *Oral Histology*. 7th ed. St Louis: Mosby Inc; 2008.
140. Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, et al. Mesenchymal stem cell-mediated functional tooth regeneration in swine. *PLoS One* 2006;1:e79.
141. Huang GT, Sonoyama W, Liu Y, Liu H, Wang S, Shi S. The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering. *J Endod* 2008;34(6):645-651.
142. Murray PE, Garcia-Godoy F, Hargreaves KM. Regenerative endodontics: a review of current status and a call for action. *J Endod* 2007;33(4):377-390.
143. Huang GT, Lin LM. Letter to the editor: comments on the use of the term "revascularization" to describe root regeneration. *J Endod* 2008;34(5):511; author reply 511-512.
144. Trevino EG, Patwardhan AN, Henry MA, Perry G, Dybdal-Hargreaves N, Hargreaves KM, et al. Effect of irrigants on the survival of human stem cells of the apical papilla in a platelet-rich plasma scaffold in human root tips. *J Endod* 2011;37(8):1109-1115.
145. Ostby BN. The role of the blood clot in endodontic therapy. An experimental histologic study. *Acta Odontol Scand* 1961;19:324-353.
146. Myers WC, Fountain SB. Dental pulp regeneration aided by blood and blood substitutes after experimentally induced periapical infection. *Oral Surg Oral Med Oral Pathol* 1974;37(3):441-450.
147. Nevins AJ, Finkelstein F, Borden BG, Laporta R. Revitalization of pulpless open apex teeth in rhesus monkeys, using collagen-calcium phosphate gel. *J Endod* 1976;2(6):159-165.
148. Iwaya SI, Ikawa M, Kubota M. Revascularization of an immature permanent tooth with apical periodontitis and sinus tract. *Dent Traumatol* 2001;17(4):185-187.

149. Banchs F, Trope M. Revascularization of immature permanent teeth with apical periodontitis: new treatment protocol? *J Endod* 2004;30(4):196-200.
150. Torabinejad M, Turman M. Revitalization of tooth with necrotic pulp and open apex by using platelet-rich plasma: a case report. *J Endod* 2011;37(2):265-268.
151. Law AS. Considerations for regeneration procedures. *J Endod* 2013;39(3 Suppl):S44-56.
152. Yamauchi N, Yamauchi S, Nagaoka H, Duggan D, Zhong S, Lee SM, et al. Tissue engineering strategies for immature teeth with apical periodontitis. *J Endod* 2011;37(3):390-397.
153. da Silva LA, Nelson-Filho P, da Silva RA, Flores DS, Heilborn C, Johnson JD, et al. Revascularization and periapical repair after endodontic treatment using apical negative pressure irrigation versus conventional irrigation plus triantibiotic intracanal dressing in dogs' teeth with apical periodontitis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2010;109(5):779-787.
154. Nakashima M, Reddi AH. The application of bone morphogenetic proteins to dental tissue engineering. *Nat Biotechnol* 2003;21(9):1025-1032.
155. Kahler B, Mistry S, Moule A, Ringsmuth AK, Case P, Thomson A, et al. Revascularization outcomes: a prospective analysis of 16 consecutive cases. *J Endod* 2014;40(3):333-338.
156. Petrino JA, Boda KK, Shambarger S, Bowles WR, McClanahan SB. Challenges in regenerative endodontics: a case series. *J Endod* 2010;36(3):536-541.
157. Iwaya S, Ikawa M, Kubota M. Revascularization of an immature permanent tooth with periradicular abscess after luxation. *Dent Traumatol* 2011;27(1):55-58.
158. Miller EK, Lee JY, Tawil PZ, Teixeira FB, Vann WF, Jr. Emerging therapies for the management of traumatized immature permanent incisors. *Pediatr Dent* 2012;34(1):66-69.
159. Shimizu E, Jong G, Partridge N, Rosenberg PA, Lin LM. Histologic observation of a human immature permanent tooth with irreversible pulpitis after revascularization/regeneration procedure. *J Endod* 2012;38(9):1293-1297.
160. Hargreaves KM, Giesler T, Henry M, Wang Y. Regeneration potential of the young permanent tooth: what does the future hold? *J Endod* 2008;34(7 Suppl):S51-56.
161. Kling M, Cvek M, Mejare I. Rate and predictability of pulp revascularization in therapeutically reimplanted permanent incisors. *Endod Dent Traumatol* 1986;2(3):83-89.

162. Laureys WG, Cuvelier CA, Dermaut LR, De Pauw GA. The critical apical diameter to obtain regeneration of the pulp tissue after tooth transplantation, replantation, or regenerative endodontic treatment. *J Endod* 2013;39(6):759-763.
163. Thibodeau B, Teixeira F, Yamauchi M, Caplan DJ, Trope M. Pulp revascularization of immature dog teeth with apical periodontitis. *J Endod* 2007;33(6):680-689.
164. Grossman LI. [Success in root canal therapy]. *Rev Odontol (B Aires)* 1954;42(6):221-225.
165. Senia ES, Marshall FJ, Rosen S. The solvent action of sodium hypochlorite on pulp tissue of extracted teeth. *Oral Surg Oral Med Oral Pathol* 1971;31(1):96-103.
166. Mohammadi Z. Sodium hypochlorite in endodontics: an update review. *Int Dent J* 2008;58(6):329-341.
167. Zehnder M, Kosicki D, Luder H, Sener B, Waltimo T. Tissue-dissolving capacity and antibacterial effect of buffered and unbuffered hypochlorite solutions. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002;94(6):756-762.
168. Ring KC, Murray PE, Namerow KN, Kuttler S, Garcia-Godoy F. The comparison of the effect of endodontic irrigation on cell adherence to root canal dentin. *J Endod* 2008;34(12):1474-1479.
169. Martin DE, De Almeida JF, Henry MA, Khaing ZZ, Schmidt CE, Teixeira FB, et al. Concentration-dependent effect of sodium hypochlorite on stem cells of apical papilla survival and differentiation. *J Endod* 2014;40(1):51-55.
170. Buck RA, Cai J, Eleazer PD, Staat RH, Hurst HE. Detoxification of endotoxin by endodontic irrigants and calcium hydroxide. *J Endod* 2001;27(5):325-327.
171. Safavi KE, Nichols FC. Effect of calcium hydroxide on bacterial lipopolysaccharide. *J Endod* 1993;19(2):76-78.
172. Siqueira JF, Jr., Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. *Int Endod J* 1999;32(5):361-369.
173. Safavi KE, Spangberg LS, Langeland K. Root canal dentinal tubule disinfection. *J Endod* 1990;16(5):207-210.
174. Sjogren U, Figdor D, Spangberg L, Sundqvist G. The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. *Int Endod J* 1991;24(3):119-125.
175. Hargreaves KM, Diogenes A, Teixeira FB. Treatment options: biological basis of regenerative endodontic procedures. *J Endod* 2013;39(3 Suppl):S30-43.

176. Han B, Wang X, Liu J, Liang F, Qu X, Yang Z, et al. Influence of calcium hydroxide-loaded microcapsules on osteoprotegerin and receptor activator of nuclear factor kappa B ligand activity. *J Endod* 2014;40(12):1977-1982.
177. Yassen GH, Vail MM, Chu TG, Platt JA. The effect of medicaments used in endodontic regeneration on root fracture and microhardness of radicular dentine. *International Endodontic Journal* 2013;46(7):688-695.
178. Yassen GH, Chu TM, Eckert G, Platt JA. Effect of medicaments used in endodontic regeneration technique on the chemical structure of human immature radicular dentin: an in vitro study. *J Endod* 2013;39(2):269-273.
179. Vijayaraghavan R, Mathian VM, Sundaram AM, Karunakaran R, Vinodh S. Triple antibiotic paste in root canal therapy. *J Pharm Bioallied Sci* 2012;4(Suppl 2):S230-233.
180. Sato I, Ando-Kurihara N, Kota K, Iwaku M, Hoshino E. Sterilization of infected root-canal dentine by topical application of a mixture of ciprofloxacin, metronidazole and minocycline in situ. *Int Endod J* 1996;29(2):118-124.
181. Sato T, Hoshino E, Uematsu H, Noda T. In vitro antimicrobial susceptibility to combinations of drugs on bacteria from carious and endodontic lesions of human deciduous teeth. *Oral Microbiol Immunol* 1993;8(3):172-176.
182. Kim JH, Kim Y, Shin SJ, Park JW, Jung IY. Tooth discoloration of immature permanent incisor associated with triple antibiotic therapy: a case report. *J Endod* 2010;36(6):1086-1091.
183. Tanase S, Tsuchiya H, Yao J, Ohmoto S, Takagi N, Yoshida S. Reversed-phase ion-pair chromatographic analysis of tetracycline antibiotics. Application to discolored teeth. *J Chromatogr B Biomed Sci Appl* 1998;706(2):279-285.
184. Zhao S, Sloan AJ, Murray PE, Lumley PJ, Smith AJ. Ultrastructural localisation of TGF-beta exposure in dentine by chemical treatment. *Histochem J* 2000;32(8):489-494.
185. Begue-Kirn C, Smith AJ, Ruch JV, Wozney JM, Purchio A, Hartmann D, et al. Effects of dentin proteins, transforming growth factor beta 1 (TGF beta 1) and bone morphogenetic protein 2 (BMP2) on the differentiation of odontoblast in vitro. *Int J Dev Biol* 1992;36(4):491-503.
186. Roberts-Clark DJ, Smith AJ. Angiogenic growth factors in human dentine matrix. *Arch Oral Biol* 2000;45(11):1013-1016.



187. Hu X, Ling J, Gao Y. Effects of irrigation solutions on dentin wettability and roughness. *J Endod* 2010;36(6):1064-1067.
188. Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, et al. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *J Endod* 2008;34(2):166-171.
189. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, et al. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci U S A* 2003;100(10):5807-5812.
190. Gronthos S, Mankani M, Brahimi J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* 2000;97(25):13625-13630.
191. Seo BM, Miura M, Sonoyama W, Coppe C, Stanyon R, Shi S. Recovery of stem cells from cryopreserved periodontal ligament. *J Dent Res* 2005;84(10):907-912.
192. Morsczeck C, Gotz W, Schierholz J, Zeilhofer F, Kuhn U, Mohl C, et al. Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. *Matrix Biol* 2005;24(2):155-165.
193. Gronthos S, Brahimi J, Li W, Fisher LW, Cherman N, Boyde A, et al. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002;81(8):531-535.
194. Smith AJ. Vitality of the dentin-pulp complex in health and disease: growth factors as key mediators. *J Dent Educ* 2003;67(6):678-689.
195. Huang GT, Yamaza T, Shea LD, Djouad F, Kuhn NZ, Tuan RS, et al. Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. *Tissue Eng Part A* 2010;16(2):605-615.
196. Torabinejad M, Faras H. A clinical and histological report of a tooth with an open apex treated with regenerative endodontics using platelet-rich plasma. *J Endod* 2012;38(6):864-868.
197. Jadhav G, Shah N, Logani A. Revascularization with and without platelet-rich plasma in nonvital, immature, anterior teeth: a pilot clinical study. *J Endod* 2012;38(12):1581-1587.
198. Shivashankar VY, Johns DA, Vidyanath S, Kumar MR. Platelet Rich Fibrin in the revitalization of tooth with necrotic pulp and open apex. *J Conserv Dent* 2012;15(4):395-398.

199. Du T, Wang Z, Shen Y, Ma J, Cao Y, Haapasalo M. Effect of long-term exposure to endodontic disinfecting solutions on young and old *Enterococcus faecalis* biofilms in dentin canals. *J Endod* 2014;40(4):509-514.
200. Wang Z, Shen Y, Haapasalo M. Effectiveness of endodontic disinfecting solutions against young and old *Enterococcus faecalis* biofilms in dentin canals. *J Endod* 2012;38(10):1376-1379.
201. Ghaeth Yassen, George Eckert, Platt J. Effect of intracanal medicaments used in endodontic regeneration procedures on microhardness and chemical structure of dentin. *Restorative Dentistry and Endodontics* 2014(2234-7666):1-9.
202. Diogenes A, Henry MA, Teixeira FB, Hargreaves KM. An update on clinical regenerative endodontics. *Endod Top* 2013;28(1):2-23.
203. Althumairy RI, Teixeira FB, Diogenes A. Effect of dentin conditioning with intracanal medicaments on survival of stem cells of apical papilla. *J Endod* 2014;40(4):521-525.
204. Labban N, Yassen GH, Windsor LJ, Platt JA. The direct cytotoxic effects of medicaments used in endodontic regeneration on human dental pulp cells. *Dent Traumatol* 2014;30(6):429-434.
205. Yassen GH, Chu TM, Eckert G, Platt JA. Effect of medicaments used in endodontic regeneration technique on the chemical structure of human immature radicular dentin: an in vitro study. *J Endod* 2013;39(2):269-273.
206. Yassen GH, Sabrah AH, Eckert GJ, Platt JA. Effect of different endodontic regeneration protocols on wettability, roughness, and chemical composition of surface dentin. *J Endod* 2015;41(6):956-960.
207. Sabrah AH, Yassen GH, Spolnik KJ, Hara AT, Platt JA, Gregory RL. Evaluation of Residual Antibacterial Effect of Human Radicular Dentin Treated with Triple and Double Antibiotic Pastes. *J Endod* 2015;41(7):1081-1084.
208. Sabrah AH, Yassen GH, Liu WC, Goebel WS, Gregory RL, Platt JA. The effect of diluted triple and double antibiotic pastes on dental pulp stem cells and established *Enterococcus faecalis* biofilm. *Clin Oral Investig* 2015;19(8):2059-2066.
209. Prather BT, Ehrlich Y, Spolnik K, Platt JA, Yassen GH. Effects of two combinations of triple antibiotic paste used in endodontic regeneration on root microhardness and chemical structure of radicular dentine. *J Oral Sci* 2014;56(4):245-251.

210. Sabrah AH, Yassen GH, Gregory RL. Effectiveness of antibiotic medicaments against biofilm formation of *Enterococcus faecalis* and *Porphyromonas gingivalis*. *J Endod* 2013;39(11):1385-1389.
211. Berkhoff JA, Chen PB, Teixeira FB, Diogenes A. Evaluation of triple antibiotic paste removal by different irrigation procedures. *J Endod* 2014;40(8):1172-1177.
212. Mohammadi Z, Giardino L, Mombeinipour A. Antibacterial substantivity of a new antibiotic-based endodontic irrigation solution. *Aust Endod J* 2012;38(1):26-30.
213. Khademi AA, Mohammadi Z, Havaee A. Evaluation of the antibacterial substantivity of several intra-canal agents. *Aust Endod J* 2006;32(3):112-115.
214. Blanscet ML, Tordik PA, Goodell GG. An agar diffusion comparison of the antimicrobial effect of calcium hydroxide at five different concentrations with three different vehicles. *J Endod* 2008;34(10):1246-1248.
215. Behnen MJ, West LA, Liewehr FR, Buxton TB, McPherson JC, 3rd. Antimicrobial activity of several calcium hydroxide preparations in root canal dentin. *J Endod* 2001;27(12):765-767.
216. Kurosawa H. Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. *J Biosci Bioeng* 2007;103(5):389-398.
217. Sabrah AH. Diluted antibiotics for treating traumatized immature teeth [Dissertation]. Indiana University School of Dentistry; 2014.
218. Tagelsir A, Yassen GH, Gomez GF, Gregory RL. Effect of Antimicrobials Used in Regenerative Endodontic Procedures on 3-week-old *Enterococcus faecalis* Biofilm. *J Endod* 2015.
219. Ordinola-Zapata R, Bramante CM, Cavenago B, Graeff MS, Gomes de Moraes I, Marciano M, et al. Antimicrobial effect of endodontic solutions used as final irrigants on a dentine biofilm model. *Int Endod J* 2012;45(2):162-168.
220. Shin SY, Albert JS, Mortman RE. One step pulp revascularization treatment of an immature permanent tooth with chronic apical abscess: a case report. *Int Endod J* 2009;42(12):1118-1126.
221. McCabe P. Revascularization of an immature tooth with apical periodontitis using a single visit protocol: a case report. *Int Endod J* 2015;48(5):484-497.
222. Fouad AF, Nosrat A. Pulp regeneration in previously infected root canal space. *Endod Top* 2013;28(1):24-37.

223. Fouad AF, Verma P. Healing after regenerative procedures with and without pulpal infection. *J Endod* 2014;40(4 Suppl):S58-64.
224. Porter ML, Munchow EA, Albuquerque MT, Spolnik KJ, Hara AT, Bottino MC. Effects of Novel 3-dimensional Antibiotic-containing Electrospun Scaffolds on Dentin Discoloration. *J Endod* 2016;42(1):106-112.
225. Akcay M, Arslan H, Yasa B, Kavrik F, Yasa E. Spectrophotometric analysis of crown discoloration induced by various antibiotic pastes used in revascularization. *J Endod* 2014;40(6):845-848.
226. Reynolds K, Johnson JD, Cohenca N. Pulp revascularization of necrotic bilateral bicuspid using a modified novel technique to eliminate potential coronal discoloration: a case report. *Int Endod J* 2009;42(1):84-92.
227. Park HB, Lee BN, Hwang YC, Hwang IN, Oh WM, Chang HS. Treatment of non-vital immature teeth with amoxicillin-containing triple antibiotic paste resulting in apexification. *Restor Dent Endod* 2015;40(4):322-327.

**ABSTRACT**

# The longevity of residual antibacterial effect of dentin treated with various concentrations of triple antibiotic paste

by

Sarmad Mazin Alyas

Indiana University School of Dentistry  
Indianapolis, Indiana

**Introduction:** Triple antibiotic paste (TAP, 1000 mg/ml) is composed of equal portions of ciprofloxacin, metronidazole and minocycline and is used as an intracanal dressing to disinfect the infected immature root canal during endodontic regeneration procedures. Lower concentrations of TAP have been recommended to minimize detrimental effects on pulp stem cells. TAP can be retained within the dentin matrix and its continual release confers an antibacterial effect to the dentin. **Objective:** The aim of this *in vitro* study was to investigate the residual antibacterial effect of dentin treated with various concentrations of TAP loaded into a gel system. **Materials and Methods:** Radicular dentin slabs were prepared from human teeth after obtaining IRB approval. The slabs were sterilized and treated with methylcellulose-based TAP of 100 mg/mL, 10 mg/mL, 1 mg/mL, 1.5% NaOCl, placebo paste with no TAP, or a positive control group with pure 1000 mg/mL TAP. Samples in each group were treated with the assigned TAP concentration for three weeks or immersed in 1.5% NaOCl for five minutes (n =18 per group). All samples were then irrigated with sterile water followed by 17% EDTA and incubated in phosphate buffered saline for either 2 or 4 weeks. Samples were then

inoculated with *Enterococcus faecalis* and incubated for an additional 3 weeks. Biofilm formed on each sample was then dislodged and spiral plated to evaluate the bacterial colony-forming units. Data were analyzed using Fisher's Exact tests and Wilcoxon rank sum tests ( $\alpha = 0.05$ ). **Results:** Dentin treated with 10, 100, or 1000 mg/mL of TAP demonstrated significant residual antibacterial effects up to four weeks. However, only 100 mg/mL TAP was able to completely prevent bacterial colonization after four weeks. No considerable residual antibacterial effect was observed in dentin treated with placebo gel, 1 mg/ml TAP or 1.5% NaOCl. **Conclusion:** At least 10 mg/mL of TAP loaded into a methylcellulose system is required to achieve a substantial residual antibacterial effect for four weeks.

## **CURRICULUM VITAE**



## **Sarmad Mazin Alyas (Askar)**

2054 Kingsway Dr.

Troy, MI 48098

March 10 <sup>th</sup> 1984	Born to Nada Hasso & Mazin Askar, Baghdad, Iraq
2003 - 2007	Bachelor of Science (BS) Calvin College Grand Rapids, MI
2007 - 2009	Master of Science (MS) Wayne State University Detroit, MI
2009 – 2013	Doctor of Dental Surgery (DDS) University of Detroit Mercy School of Dentistry Detroit, MI
2013 – 2014	General Practice Residency Certificate Summa Health System Akron, OH
2014 - 2016	Endodontic Resident & Graduate Student Indiana University School of Dentistry Indianapolis, IN
June 30, 2016	MSD and Certificate in Endodontics Indiana University School of Dentistry Indianapolis, IN

### **Professional Societies**

American Association of Endodontists (AAE)

American Dental Association (ADA)

Michigan Association of Endodontists (MAE)

Michigan Dental Association (MDA)